Statin Resistance and Export

- development of a yeast cell factory for compactin

Ley, Ana

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- Development of a Yeast Cell Factory for Compactin Production

Ana Ley

Technical University of Denmark, Department of Systems Biology

May 2015, Kongens Lyngby, Denmark

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Supervisors:
Assistant Professor Rasmus John Normand Frandsen, DTU
Professor Uffe Hasbro Mortensen, DTU

Evaluation Committee/Examiners:
Professor Colin Lazarus, University of Bristol
Professor Barbara Ann Halkier, University of Copenhagen
Associate Professor Ulf Thrane, DTU

Date of defense: 30th of June 2015

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- Development of a Yeast Cell Factory for Compactin Production

Ana Ley

Address: Department of Systems Biology
Technical University of Denmark
Building 223
2800 Kgs. Lyngby
Denmark

Phone: (+45) 4525 2600
Fax: (+45) 4588 4922
Web: http://www.bio.dtu.dk/english

Preface

This thesis serves as a partial fulfillment of the requirements to obtain the PhD degree at Technical University of Denmark (DTU). The PhD project was initiated in January 2012, and was carried out at DTU Department of Systems Biology under supervision of assistant professor Rasmus John Normand Frandsen and professor Uffe Hasbro Mortensen.

Thank you Lučka Drobnič Thurnherr for your immense contribution to my personal and professional development. For showing me the mountains and climbing them with me! Thank you Daniel Ley for walking this path with me, with love and understanding.

Abstract

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the key enzyme in the mevalonate pathway that leads to the synthesis of cholesterol and ergosterol in animal and fungal cells, respectively. Their extensive use in treatment and prevention of cardiovascular diseases placed statins among the best-selling pharmaceuticals. Industrial scale production of natural statins (i.e. compactin and lovastatin) and their semi-synthetic derivatives (i.e. pravastatin and simvastatin) is based on fermentation of statin-producing filamentous fungi, such as *Penicillium sp*. and *Aspergillus sp*.. Production limitations associated with the unique physiology and morphology of the natural producers can be overcome by heterologous expression of the pathway in a fast-growing host, such as *S. cerevisiae*. Future construction of *S. cerevisiae* cell factory for the production of high concentrations of natural statins will require the establishment of a non-destructive self-resistance mechanism to overcome the undesirable growth inhibition effects of statins. In an effort to resolve this challenge, two putative self-resistance genes, *mlcD* and *mlcE*, originating from the *P. citrinum* compactin gene cluster, were tested for their ability to protect yeast from statins. Chromosomal gene integration approach was used to express the genes in *S. cerevisiae*.

This study showed that *mlcD* could mediate statin-resistance when expressed heterologously in *S. cerevisiae,* increasing yeast resistance from 0.25 mM to at least 1.24 mM. Successful complementation of *Sc-HMG1* and *Sc-HMG2* in yeast, in addition, proved that MlcD functions as HMGCR. A phylogenetic analysis of fungal HMGCRs revealed that HMGCRs from the known statin gene clusters (*mlcD and lvrA/mokG*) are likely derived from HMGCRs involved in primary metabolism. However, the occurrence of these genes in the different statin gene clusters probably did not arise from a recent duplication of the primary HMGCR in the producing organism. A model was proposed, in which the HMGCR-encoding

genes at some time during evolution were duplicated and then recruited to the statin gene clusters, a situation that has increased the chance for becoming co-regulated with the cluster and hence statin production. Collectively these results suggest that statin self-resistance is based on the HMGRS's association with the cluster, e.g. increased concentration of HMGCR at the right time, and is not due to the HMGCR being statin-insensitive. This model will require further validation by e.g. direct measurement of enzymes sensitivity to statins.

Heterologous expression of *mlcE,* the second putative self-resistance gene from the compactin gene clsuter, significantly increased *S. cerevisiae's* resistance to natural statins (8.6-fold increase in growth efficiency compared to the wild-type strain). Sequence based analysis showed that MlcE likely contains 14 transmembrane spanning domains and phylogenetically it clusters together with known toxin efflux pumps from both fungi and bacteria. RFP-tagging of MlcE showed that it was localized to the plasma and vacuolar membranes in yeast. Collectively these results indicate that *mlcE* encodes for a transmembrane transporter, and thus likely provides the resistance to statins by secreting the compounds outside of the cells. Further testing of MlcE's role as a self-resistance mechanism surprisingly showed that it was not only capable of protecting yeast from the negative effects of natural statins, but also semi-synthetic statins (i.e simvastatin).

This work provides possible engineering strategies for improvement of future yeast based production of natural and semi-synthetic statins in yeast. Moreover, it gives new insights into the statin self-resistance mechanisms in the natural producers.

Sammenfatning

Statiner er inhibitorer af 3-hydroxy-3-methylglutaryl coenzym A reduktase (HMGCR), det vigtigste enzym i mevalonat biosyntesevejen, der er ansvarlig for produktion af henholdsvis kolesterol og ergosterol i dyre- og svampeceller. Deres omfattende brug til forebyggelse og behandling af cholestrol forårsagede hjerte-kar sygdomme har betydet at statiner er blandt de bedst sælgende lægemidler. Produktion af naturlige statiner (compactin og lovastatin) og deres semisyntetiske derivater (pravastatin og simvastatin) i industriel skala er baseret på fermentering af naturligt statin-producerende skimmelsvampe som *Penicillium* og *Aspergillus*. De naturlige producenters unikke fysiologi og morfologi udgør i dag en begrænsning for den industrielle produktion, men disse begrænsninger forventes at kunne omgås ved at udtrykke biosyntesevejen heterologt i en hurtigt groende vært som *Saccharomyces cerevisiae* (gær). Konstruktion af en højt ydende *S. cerevisiae* cellefabrik, til produktion af naturlige statiner, vil dog kræve at der udvikles en effektiv og nedbrydnings-uafhængig resistensmekanisme der kan modvirke den uønskede væksthæmmende effekt statiner har på gærceller. I et forsøg på at identificere løsninger på denne problemstilling, blev to formodede resistens kodende gener, *MlcD* og *MlcE* fra compactin gen-clusteret i *P. citrinum,* testet for deres evne til at beskytte gærceller mod statiners negative effekter. Genernes duelighed blev testet ved at integrere dem kromosomalt i *S. cerevisiae*.

Det gennemførte studie demonstrerede at heterolog ekspression af *MlcD* i *S. cerevisiae* forøgede gærs resistensniveau for compactin fra 0.25 mM til mindst 1.24 mM. Derudover viste en komplementeringsanalyse af *Sc-HMG1* og *Sc-HMG2* mutationerne i gær, at MlcD fungerer som en HMGCR, da den kunne reversere begge mutanter til vild-type fænotypen. En fylogenetisk analyse af svampe HMGCR enzymer afslørede at HMGCR'er der koder for gener fra de kendte statin producerende genclustre (*mlcD* og *lvrA*/*mokG*) sandsynligvis stammer fra HMGCR

involveret i primær metabolisme. Tilstedeværelsen af disse gener i de forskellige genclustre, der er ansvarlige for statin produktion, er formentlig ikke resultatet af en nylig genduplikation af den primære HMGCR i de producerende svampe. En model der muligvis kan forklare den nuværende situation er, at de HMGCR-kodende gener på et tidspunkt i evolutionen blev duplikeret og derefter rekrutteret til statin-genclustrene; en situation der har forøget sandsynligheden for at deres ekspressionsmønster er blevet co-reguleret med clusteret, og dermed statin-produktionen. Samlet set betyder dette, at opståen af HMGCR baseret statinresistens er associeret med rekruttering af de HMGCR kodende gener til statin genclustrene, hvilket har øget koncentrationen af HMGCR i statin-producerede celler; dette gør modeller hvor statinresistens skyldes udviklingen af statin insensitive HMGCR i forbindelse med statin-clusterne mindre sandsynlige. Denne model kræver yderligere validering via for eksempel direkte måling af enzymernes resistens overfor statin-inhibering.

Heterolog udtryk af *MlcE*, det andet formodede resistensgen fra compactin genclusteret, forøgede *S. cerevisiae*'s resistens mod naturlige statiner signifikant (8.6-fold forøget biomasseudbytte sammenlignet med vildtypen). Sekvensbaseret analyse viste, at *MlcE* formentlig indeholder 14 transmembrane domæner og rent fylogenetisk grupperer den med kendte toksin udstøms-pumper fra både skimmelsvampe og bakterier. RFP-mærkning af MlcE demonstrerede at det er lokaliseret nær plasma- og vakuolemembraner i gær. Samlet indikerer disse resultater at *MlcE* koder for et transmembrant transportprotein, og at det afstedkommer statin-resistens ved at pumpe statinerne ud af cellen. Yderligere analyser af MlcE's rolle som resistensmekanisme viste overraskende nok at den ikke kun er i stand til at beskytte gær mod de negative effekter af naturlige statiner, men også semi-syntetiske statiner (simvastatin).

Dette projekt har tilvejebragt to eksperimentelle strategier der vil muliggøre forbedring af fremtidige gær-baserede cellefabrikker til produktion af naturlige og

semi-syntetiske stationer i gær. Derudover, giver det nye indsigter i statin-producerende skimmelsvampes naturlige resistensmekanismer overfor statiner.

List of Publications

A. Ley, R. J. N. Frandsen. *"MlcD – a metabolic backup system to the standard 3-hydroxy-3-methylglutaryl coenzyme A reductase in statin producing fungi".*
Manuscript in preparation.
(Presented as Chapter 5)

A. Ley, H. C. Coumou, R. J. N. Frandsen. "*Heterologous expression of MlcE in Saccharomyces cerevisiae provides resistance to natural and semi-synthetic statins".*
Manuscript submitted to Metabolic Engineering Communications.
(Presented as Chapter 6)

Patent

Statin Resistance and Export
Inventors: **Ana Rems** and Rasmus John Normand Frandsen
Filed April 23, 2014, Europe 14165696.7-1419
Current status: PCT phase
(Presented in the Appendix)

Contributions to Conferences

Poster
A. Rems, R. J. N. Frandsen. "*Development of a Yeast Cell Factory for Compactin production and derivatization".* 7th Danish Conference on Biotechnology and Molecular Biology. May 2012, Vejle, Denmark.

Oral Presentation and Poster
A. Rems, R. J. N. Frandsen. "*Molecular Biological basis for statin resistance in naturally statin-producing organisms".* 27th Fungal Genetics Conference. March 2013, Asilomar, Pacific Grove, CA, USA.

Poster
A. Ley, R. J. N. Frandsen. "*The efflux pump MlcE from the Penicillium solitum compactin biosynthetic gene cluster increases Saccharomyces cerevisiae resistance to natural statins".* Yeast Genetics Meeting. July 2014, Seattle, WA, USA.

Oral Presentation
A. Ley, R. J. N. Frandsen. "*The efflux pump MlcE from the Penicillium solitum compactin biosynthetic gene cluster increases Saccharomyces cerevisiae resistance to natural statins".* Nordic Yeast Research Community Meeting. September 2014, Copenhagen, Denmark.

List of Abbreviations

5-FOA	5-fluororotic acid
6-MSA	6-methylsalicylic acid
ABC	ATP-binding cassette
Acetyl-CoA	Acetyl coenzyme A
ACP	Acyl carrier protein
AT	Acyltransferase
CD	Catalytic domains
CFP	Cercosporin facilitator protein
CHD	Coronary heart disease
CYC	Cyclase
DH	Dehydratase
DHA12	Drug:H+ antiporter 12
DIC	Differential interference contrast (microscopy)
DMB-SMMP	α-dimethylbutyryl-S-methyl-mercaptopropionate
DTU	Technical University of Denmark
EF	Elongation factor
ER	Enoyl reductase
FAS	Fatty acid synthase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme
HMGCR	HMG-CoA reductase
IMP	Inosine-5-monophosphate
IMPD	Inosine-5`-monophosphate dehydrogenase
IPN	Isopenicillin N
JGI	Joint Genome Institute
KR	Ketoreductase
KS	Ketoacyl-CoA synthase
LB	Lysogeny broth
LC-MS	Liquid chromatography – mass spectrometry
MFS	Major facilitator superfamily
MPA	Mycophenolic acid
MT	Methyltransferase

NBD	Nucleotide binding domains
NCBI	National Center for Biotechnology Information
NRPS	Nonribosomal peptide-synthase
P-pant	4'-phosphopantetheine
PCR	Polymerase chain reaction
PKS	Polyketide synthase
PL	Pentalenolactone
PPTase	4'-phosphopantetheinyl transferase
PT	Product template
RFP	Red fluorescent protein
ROS	Reactive oxygen species
SAM	S-adenosylmethionine
SAT	Starter acyltransferase
SC	Synthetic complete
SS	Sterol sensing
SSF	Solid state fermentation
TE	Thioesterase
TMS	Transmembrane domains
USER	Uracil-specific excision reagent
WHO	World Health Organization
WT	Wild type
YPD	Yeast peptone dextrose

Table of Content

Introduction

Coronary heart disease (CHD) is the leading cause of death according to the World Health Organization. Today, the treatment and prevention of CHD is primarily based on lowering the patients plasma cholesterol levels, as high concentration of cholesterol in the blood (hypercholesterolemia) can result in abnormal deposition of cholesterol and its ester in the walls of arteries, eventually leading to the buildup of plaques (atherosclerosis) that can completely block the arteries or rupture and cause a heart attack or a stroke. First choice of treatment and prevention of CHD is a combination of diet changes and a therapy with statins, a group of pharmaceuticals that reduces the plasma cholesterol levels by inhibiting the key enzyme in the cholesterol biosynthesis, namely HMG-CoA reductase. Due to the vast number of patients with elevated plasma cholesterol levels, statins are commercially extremely successful, and the competition with generic versions of statins is tough. The challenge of the pharmaceutical industry is not just in the discovery of novel statins, with improved pharmacokinetic and pharmacodynamic properties, but also in ensuring the availability of statins in the market at the best possible price.

The emergence of synthetic statins (e.g. atorvastatin) has not diminished the importance of biotechnological production of the natural (i.e. compactin – also called mevastatin or ML-236B – and lovastatin) and semi-synthetic statins (i.e. pravastatin and simvastatin) using fermentation-based processes. In fact, statins of microbial origin remain the first-line treatment of choice for patients with high plasma cholesterol and CHD. Industrial biotechnology, with its potential to improve the biological production of statins, is therefore indispensable.

In general, two main approaches are exploited for improving the biotechnological production of biopharmaceuticals. The first approach involves the production technology, where optimization of cultivation conditions and purification

processes can significantly improve the production yield of the desired compound. The second approach is based on the improvement of the production organism, and includes engineering of a native host, or the use and engineering of a heterologous host organism. Industrial scale production of natural and semi-synthetic statins is based on fermentation of statin-producing filamentous fungi. Optimization of bioprocessing conditions, as well as improvement of the natural producers aimed at increased production yields have been exploited extensively. In contrary, the use of heterologous hosts for the production of statins remains largely unexplored, however some of the production limitations associated with the unique physiology and morphology (e.g. filamentous growth) of the natural producers can be overcome by heterologous expression of the biosynthetic pathway in a fast-growing host, such as *Saccharomyces cerevisiae*.

My PhD project focused on the use of *S. cerevisiae* as a cell factory for the production of natural and semi-synthetic statins. The objective of my thesis work was to develop a *S. cerevisiae* based cell factory for the production of compactin, one of the two known natural statins that is produced as a secondary metabolite by the filamentous fungus *Penicillium citrinum*. I aimed at heterologous expression of the compactin biosynthetic pathway from *P. citrinum* in *S. cerevisiae* by using available genetic tools that allow for stable integration of multiple genes into the yeast genome. The second aim of this project concerned a statin resistance mechanism; if biologically active compounds, such as statins, are to be heterologously produced, an important consideration is the need for a resistance mechanism to protect the heterologous host from any toxic effects of the product may have. Since the target of compactin, HMG-CoA reductase is essential for eukaryotes, it is also found in *S. cerevisiae*, and therefore this consideration is inevitable. Self-resistance mechanisms present in the native producers can represent a toolbox for solving the resistance problem in a heterologous host, therefore I aimed at elucidating the resistance mechanism to statins and explore its potential to confer the statin resistance in *S. cerevisiae*.

I have divided this thesis into two parts; in the first part I aim at providing the theoretical background of my PhD project, which consists of Chapters 1-3. Statins are an excellent example of the successful use of naturally occurring bioactive compounds in medicine. In Chapter 1, I present the impact of natural products to our society, and I highlight the fungal secondary metabolites as one the most important sources of biopharmaceuticals. Chapter 2 is devoted to statins, where I present their important applications, chemical and biosynthetic features, and different aspects of biotechnological production of statins. In Chapter 3 I provide an overview of the different self-resistance mechanisms that organisms possess in order to cope with the negative effects of the secondary metabolites, which they synthesize. The possibility to utilize the self-mechanisms in the engineering of a cell-factory is also discussed, and examples are provided. The second part of the thesis covers the experimental part of my project, and consists of Chapters 4-6, each representing a specific research case. The first case – an attempt to construct a *S. cerevisiae* cell factory for compactin production – is briefly described in Chapter 4. The two subsequent research cases involved elucidation of the statin resistance mechanism, and investigation its potential in *S. cerevisiae*. This part of the project concerned two different putative resistance genes from *P. citrinum* (Figure 1); in Chapter 5 I present my work that involved a putative HMG-CoA reductase-encoding gene (*mlcD*), and in Chapter 6 I describe the work associated with a putative efflux pump (*mlcE*). Chapter 6 is a research article that was submitted for publication. Moreover, the findings from Chapter 6 served as a basis for a patent, which was filed in April 2014, and which is shown as an appendix to the thesis. Overview of the structure of this thesis is presented in Figure 1.

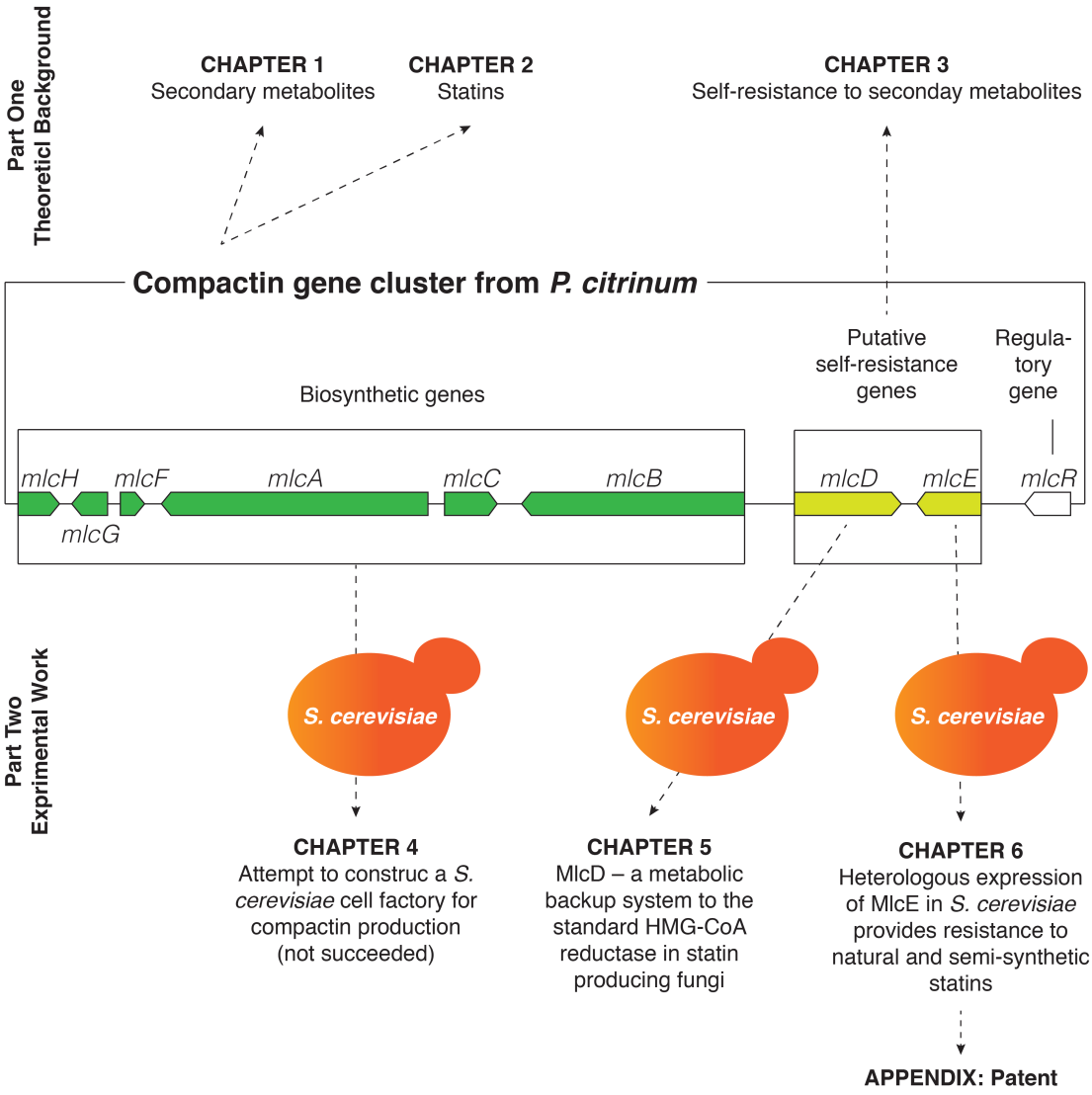

Figure 1: Overview of the thesis and research work. Chapters 1-3 provide theoretical background, and Chapters 4-6 describe the experimental work. Patent based on the work described in Chapter 6 is added as an appendix to this thesis.

PART ONE (Theoretical Background)

CHAPTER 1

Secondary metabolites

1.1 Naturally occurring bioactive molecules – secondary metabolites

Nature is an important source of novel bioactive compounds, many of which have had a tremendous impact to our society. They have reduced the pain and suffering, moved the borders of medicine by allowing organ transplantation, and are among the most important drugs for treatment of cancer and infectious diseases. Molecules originating from biological systems can be applied unmodified in medicine or any other field (e.g. agriculture), or can serve as a starting material for chemical or microbial derivatizations. Furthermore, they can be used as lead compounds for chemical synthesis of new analogs or as templates for rational drug design studies (Berdy, 2005; Newman and Cragg, 2012). Most of the naturally occurring bioactive compounds are products of secondary metabolism, which helps the organisms to meet the needs and challenges of their natural environment and lifestyle. Secondary metabolites are structurally heterogeneous, small and often complex molecules that are, in contrast to primary metabolites, not required for growth (at least under laboratory conditions), but which provide a competitive advantage to the producing organism (Williams et al., 1989). Both, higher eukaryotic forms of life, such as plants (mostly higher plants, but also lichens and algae) and animals (especially marine animals), as well as microorganisms are producing secondary metabolites, however synthesis of specific compounds is typically restricted to narrow taxonomic groups of organisms, e.g. strains within species (Challis and Hopwood, 2003).

Microorganisms are the most frequent and versatile producers of secondary metabolites (Demain, 1999). Most of the biologically active secondary metabolites have been isolated from actinomycetes (filamentous bacteria) and filamentous fungi (Bérdy, 2005). Several different biological functions have been attributed to microbial secondary metabolites, including (i) the inhibition or killing of competing organisms in the battle for the resources, (ii) metal uptake and transport, (iii) protection against abiotic stress (e.g. light), (iv) differentiation effectors etc. (Challis and Hopwood, 2003; Demain, 2000). A more general explanation of the natural function of secondary metabolites came from Bérdy (Bérdy, 2005), who defined secondary metabolites as a chemical interface between microbes and the rest of the world – the interface that is manifested in diverse interactions, e.g. antagonistic, synergistic, regulatory, modulatory, and with different living systems, e.g. other microbes, animals and plants.

To date statins have only been identified as products of filamentous fungi, and this group will therefore be introduced in the following section.

1.2 Fungal secondary metabolites

The discovery of penicillin in 1928 (Fleming, 1929) accelerated the in research of fungal metabolites, including discovery, isolation and characterization of numerous novel secondary metabolites (Bennett, 2001). Fungal secondary metabolites have proven to be an excellent source of natural products with pharmaceutical applications, ranging from antibiotics (e.g. penicillins and cephalosporins), antitumor agents (e.g. macrosporin), immunosuppressive agents (e.g. cyclosporin A and mycophenolic acid), and cholesterol-lowering agents (i.e. lovastatin and compactin). The later compounds are the molecules of interest for this thesis, and Chapter 2 is devoted to a thorough introduction to this class of compounds. In contrast to the pharmaceutically attractive secondary metabolites,

fungi also produce potent toxins, such as aflatoxins and trichothecenes (Figure 2) (Hoffmeister and Keller, 2007).

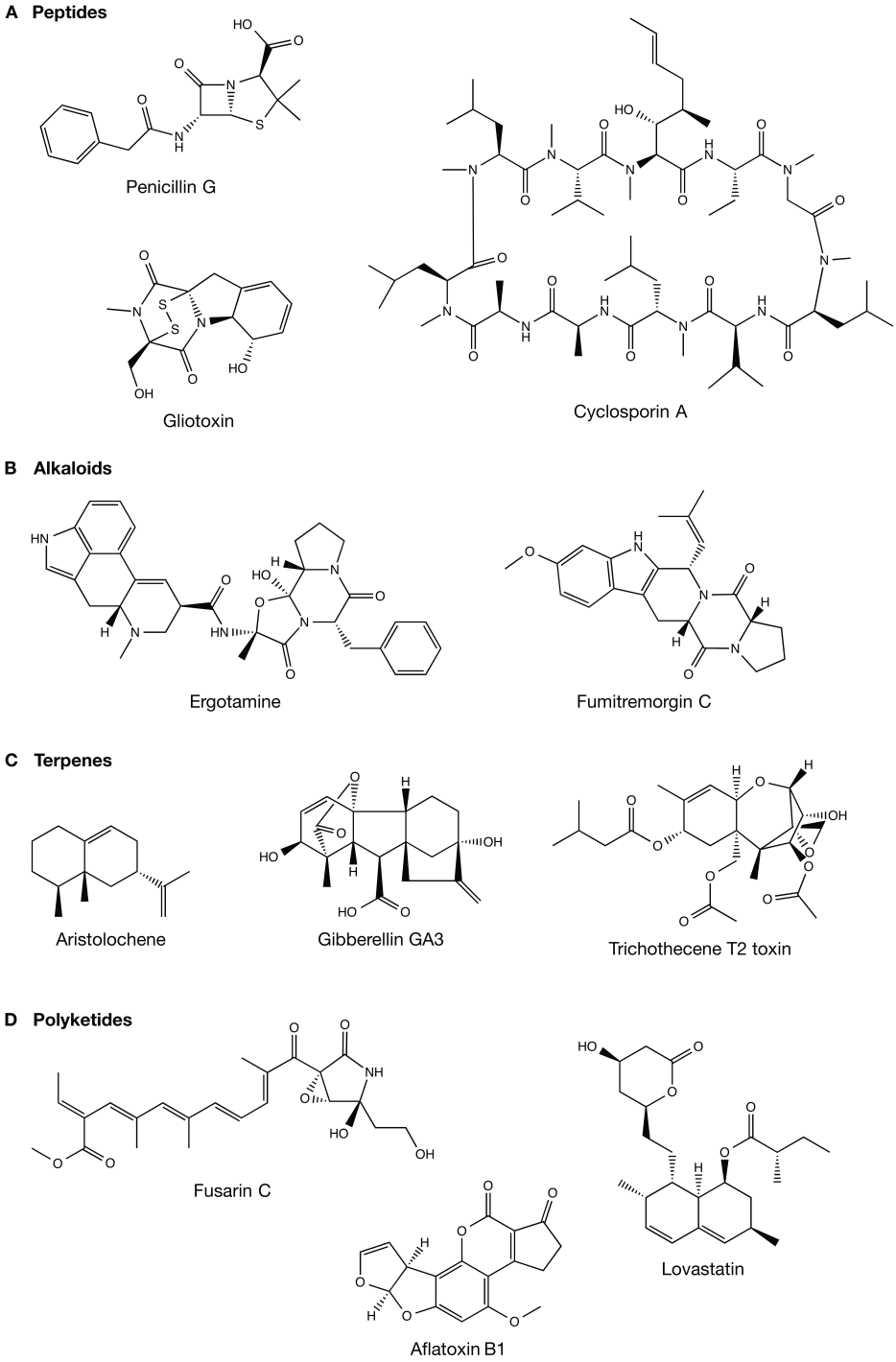

Figure 2: Examples of fungal secondary metabolites. Penicillin G from *Penicillium chrysogenum* is a clinically used antibiotic, and lovastatin from *Aspergillus terreus* is used as cholesterol-lowering compound. Cyclosporin A is immunosuppressant produced by *Tolypaocladium inflatum*. Ergotamines from *Claviceps purpurea* are anti-migraine agents. Fumitremorgin C from *A. fumigatus*, and related compounds can be used to improve chemotherapy in breast cancer treatment. Gibberellin GA3, a potent plant-growth regulator, is produced by *Fusarium fujikuroi*. Many secondary metabolites are toxic, including gliotoxin from *A. fumigatus*, aflatoxins from *A. flavus*, and trichothecenes and fusarin C from *Fusarium* species. Aristolochene from *P. roquefortii* is a precursor of a PR toxin (Brakhage, 2013; Keller et al., 2005; Sanchez et al., 2012).

1.2.1 Biosynthesis of fungal secondary metabolites

Despite their chemical diversity and complexity (Figure 2), fungal secondary metabolites are synthesized by a limited number of basic biosynthetic mechanisms, and from a limited number of precursors derived from primary metabolism (Figure 3). The enzyme classes involved in the biosynthesis of secondary metabolites form the basis for chemical classification of the metabolites into the following groups: polyketides, non-ribosomal peptides, alkaloids, terpenes, and compounds of mixed biosynthetic origin (examples of each class are presented in Figure 2), polyketides being the most abundant (Keller et al., 2005). Statins are synthesized via the polyketide pathway, thus the mechanism of polyketide biosynthesis is briefly described below.

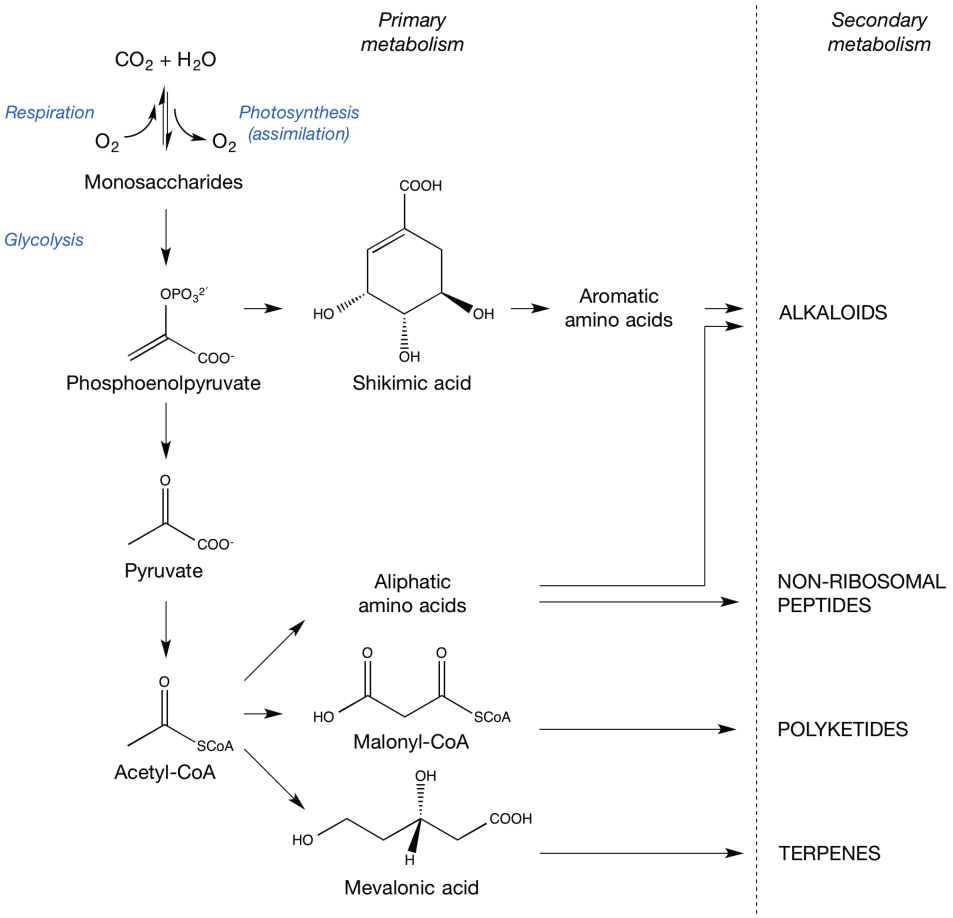

Figure 3: Secondary metabolites are derived from the precursors that originate from the primary metabolism.

1.2.2 Polyketide biosynthesis

Polyketides were first characterized as compounds with polyketomethylene groups $(CH_2\text{-}CO)_n$ (Collie, 1907). However, it is the way in which polyketides are synthesized – by polyketide synthases (PKSs) – that joins these compounds in a group (Bentley and Bennett, 1999). Fungal polyketides are usually synthesized by type I PKSs, enzymes structurally and functionally related to eukaryotic fatty-acid synthases (FASs). Both enzyme classes catalyze a Claisen condensation of primary metabolites – short-chain carboxylic acids, usually acetyl coenzyme A (acetyl-CoA) and malonyl CoA – forming carbon chains of varying lengths. Both, type I PKSs and FASs have a multi-domain structure with β-keto-synthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) domains being essential for the synthesis of the carbon backbone. Moreover, β-ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains, which are always present in FASs and are required for the reduction of the carbon backbone, are optional domains in the type I PKSs (Fujii et al., 2001). The degree of reduction of the carbon chain is the main difference between the polyketides and the fatty acids, the later being fully reduced. Other catalytic domains, affecting the structure of the polyketide have been found in fungal type I PKSs, some of which are listed in Table 1.

Table 1

Different active domains found in fungal type I PKSs and their functions (Frandsen, 2010; Fujii et al., 2001).

Domain	Function
ACP – Acyl carrier protein	Carries the growing polyketide chain as a thioester
SAT – Starter acyltransferase	Loads the starter units in the non-reducing PKSs
AT – Acyltransferase	Loads the starter, extender and intermediate units
KS – Ketoacyl-CoA synthase	Catalyzes Claisen condensation reaction
KR – Ketoreductase	Reduces ketone groups to hydroxyl groups
DH – Dehydratase	Reduces hydroxyl groups to enoyl groups
ER – Enoyl reductase	Reduces enoyl groups to alkyl groups
TE – Thioesterase	Catalyzes hydrolysis of the thioester linking the polyktetide to PKS
MT – Methyltransferase	Transfers methyl groups to the growing polyketide chain
CYC – Cyclase	Facilitates ring formation by Claisen type cyclization reaction
PT – Product template	Determines the folding pattern of the polyketide chain in non-reducing iterative PKSs

The active domains of PKSs are organized in modules, defined as a complete set of domains that are able to extend the growing polyketide chain by one ketide unit. In contrast to bacterial type I PKSs, which can consist of several modules – one for each condensation reaction – fungal type I PKSs always consist of a single module, which is able to carry out biosynthetic reactions repeatedly during formation of a single product, thus called iterative PKSs. The basic mechanism of polyketide biosynthesis by iterative PKS is illustrated in Figure 4, where the reducing steps are also shown. The high chemical diversity of fungal polyketides results from several factors; number of iterations of the condensation reaction (Figure 4, step 3), type of building blocks used for the synthesis (Figure 4, steps 1 and 2), number of reductions and cyclisation of the polyketide chain (Figure 4, steps 4-6), methylation or other branching of the polyketide chain. In addition to the factors that are “programed” by the domain structure of the PKS and influence the polyketide structure during its synthesis, post-PKS modifications can further modify the molecules, introducing additional chemical diversity. Enzymes such as oxidases, dehydrogenases, esterases, methyl-transferases, reductases, etc. – often called tailoring enzymes – carry out these modifications.

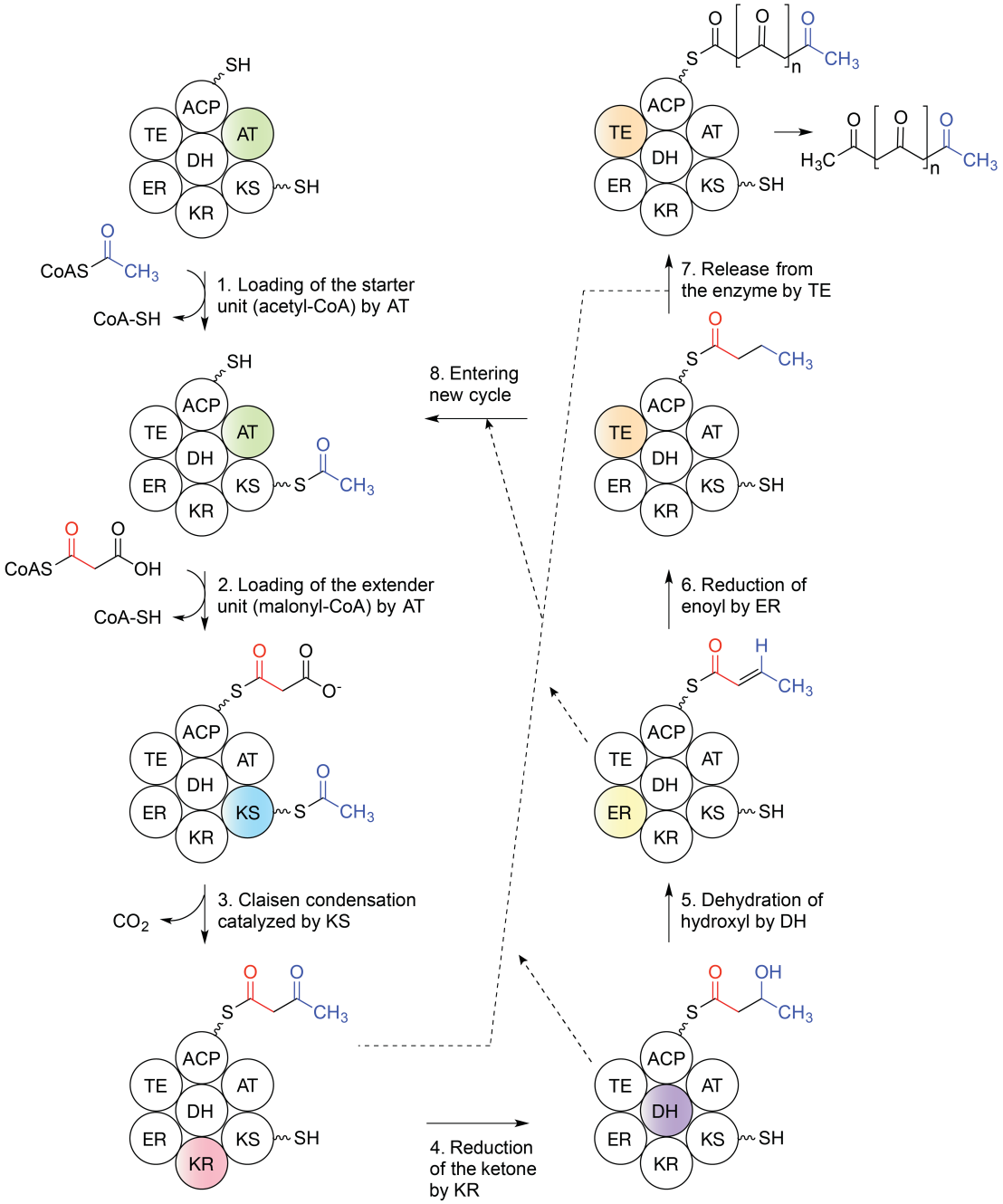

Figure 4: Mechanism of polyketide synthesis by fungal iterative type I PKS. A starter unit (acetyl-CoA in this example) and an extender unit (malonyl-CoA in this example) are loaded to the KS and ACP domain, respectively by the AT[1] domain (steps 1 and 2). The ACP domain has a flexible 4'-phosphopantetheine group. The later is able to covalently attach the substrates, intermediates and final products in a thioester linkage and move them between different active sites in the enzyme (Nelson and Cox, 2005). The KS domain catalyzes Claisen condensation between the starter and extender unit (step 3), resulting in the release of CO_2 and the formation of the carbon-carbon bond between the two units. The growing polyketide chain can then be either (i) transferred back to the KS domain to enter a new round of adding the extender unit (step 8), (ii) released from the PKS by the TE domain (step 7), or (iii) can be first reduced before it is further elongated or released. During the reducing steps the ACP presents the polyketide chain to the different reducing domains (KR, DH, ER). The reduction process can be stopped at any stage (4, 5 or 6), resulting in the different degrees of reduction of the polyketide (adapted from (Frandsen, 2010)).

[1] The AT domain is responsible for loading of the starter unit in reducing PKSs, however in the non-reducing PKSs this function is carried out by the SAT domain (Crawford et al., 2006; Foulke-Abel and Townsend, 2012).

1.2.3 Organization and regulation of fungal secondary metabolite genes

Genes encoding the biosynthetic apparatuses for the formation of secondary metabolites are usually arranged in contiguous clusters in fungal genome (Brakhage, 2013; Smith et al., 1990). Besides the genes encoding the key synthases (e.g. PKSs and non-ribosomal peptide synthase (NRPSs)), these gene clusters also encode tailoring enzymes, and regulatory proteins, most often cluster-specific transcription factors. Secondary metabolite gene clusters are not constitutively expressed; their complex regulation depends on the pathway-specific transcription factors (e.g. AflR, MlcR, etc.) as well as "global regulators" (transcription factors and regulatory networks). The global regulators are encoded by genes that do not belong to any cluster and control the response to environmental conditions such as carbon (e.g. CreA), nitogen (e.g. AreA), pH (e.g. PacC), and light (e.g. LaeA, VeA) (Brakhage, 2013; Hoffmeister and Keller, 2007). In addition to the genes encoding proteins for biosynthesis, modifications and regulation, clusters can also include genes encoding transporters and self-resistance mechanisms, a subject that is further presented in Chapter 3.

CHAPTER 2

Statins: cholesterol-lowering secondary metabolites

2.1 The use of statins as pharmaceuticals

Elevated level of cholesterol in the blood (hypercholesterolemia) can result in abnormal deposition of cholesterol and its ester in the walls of the coronary arteries, causing buildup of plaques (atherosclerosis). Such a situation can lead to a complete blockage of the arteries, and thereby heart attack or stroke. Hypercholesterolemia is known to be the primary risk factor in coronary heart disease (CHD) (KANNEL et al., 1961; Stamler, 1978), which caused one third of all deaths worldwide in 2012 according to the World Health Organization, and is considered as the leading cause of death (WHO, 2014). As approximately two-thirds of the total body cholesterol are derived from its *de novo* synthesis, mainly in the liver (Grundy and Diego, 1978), inhibition of cholesterol biosynthesis represents the most important approach to lower the plasma cholesterol levels, and thereby reduce the risk of CHD.

Cholesterol is synthesized via the mevalonate pathway (Bloch, 1957; Frantz and Schroepfer, 1967), where the rate-limiting step is the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate by the enzyme HMG-CoA reductase (HMGCR) (Rodwell et al., 1976) (Figure 5). The later therefore is a prime target for pharmacological intervention (Alberts et al., 1980). Statins are a class of pharmaceuticals that inhibit HMGCR, and thereby effectively lower the plasma cholesterol levels (Corsini et al., 1995). They have been successfully used to treat and prevent CHD, thus became one of the best-selling pharmaceuticals in past

decades. In 2012 the worldwide statin market value was estimated to 19.7 billion USD. The introduction of generic statins has temporarily meant a decline in the market value to 12.2 billion USD in 2018 (GBI Research, 2013), but it is predicted to make statin-based therapies more accessible, and the market is expected to reach 35 billion USD by 2023 (dos Santos et al., 2014). In Denmark, more than 10 % of the population receives statin therapy annually (*Fokusrapport – Viden om forbrug og bivirkninger ved behandling med statiner*, 2012)[2].

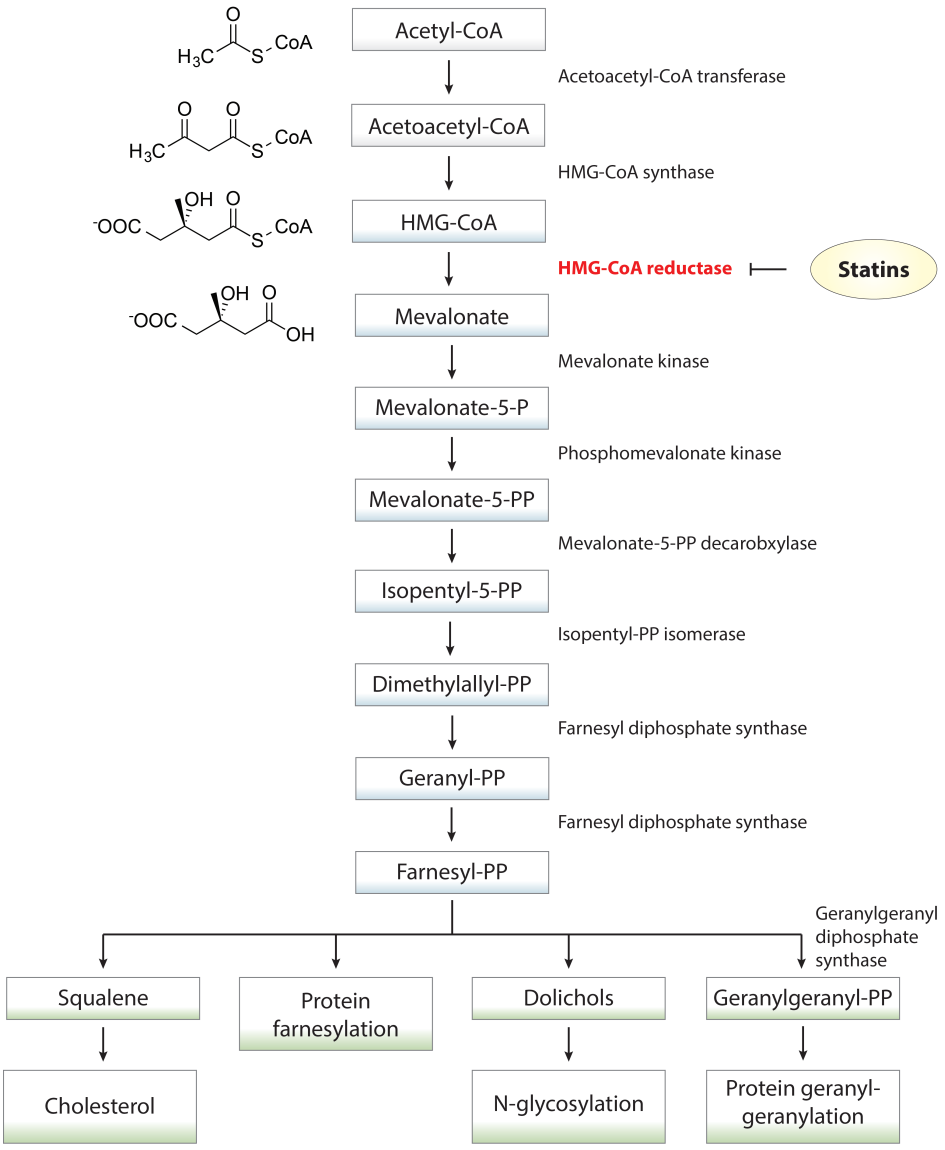

Figure 5: Schematic representation of the mevalonate pathway. Statins inhibit HMG-CoA reductase, the key enzyme in the mevalonate pathway (adapted from (Thurnher et al., 2012)).

[2] English translation: Focus Report - Knowledge of consumption and side effects of treatment with statins.

Besides their cholesterol-lowering effect, statins have been shown to have a variety of pleiotropic effects (Davignon and Leiter, 2005), and have therefore emerged as possible medicines to treat non-cardiovascular conditions, such as cancer (Gauthaman et al., 2009), dementia (Jick et al., 2014; Wolozin et al., 2000), osteoporosis (Pahan et al., 1997), multiple sclerosis (C.-Y. Wang et al., 2008a), rheumatoid arthritis (McCarey et al., 2005), and Parkinson`s disease (Becker, 2008). These pleiotropic effects are thought to be the result of a modified protein prenylation pattern. Isoprenoids derived from the mevalonate pathway are important lipid attachments of proteins involved in the intracellular signaling (Figure 5) (C.-Y. Wang et al., 2008b). More specifically, farnesyl pyrophosphate and geranylgeranyl pyrophosphate are attached to proteins during posttranslational modification, a process referred to as protein prenylation. These lipid adjuncts play a role in membrane attachment or protein-protein interactions, which can be essential for the protein functionality. Therefore it is hypothesized that inhibition of HMGCR, resulting in modulation of isoprenoid synthesis, and thereby protein prenylation, is the key mechanism by which statins interfere with the inflammatory and immune responses (Greenwood et al., 2006; Thurnher et al., 2012).

2.2 Structure and mode of inhibition

Different types of statins are available, and these are divided into three categories based on their origin; (i) natural statins (also called microbial statins (dos Santos et al., 2014)) (i.e. lovastatin and compactin), (ii) semi-synthetic statins (e.g. simvastatin and pravastatin), and (iii) statins of synthetic origin (e.g. atorvastatin, rosuvastatin, fluvastatin). All statins share an HMG-like substructure, responsible for the HMGCR inhibition activity (Istvan and Deisenhofer, 2001). The HMG-like moiety can be present in an active form (*i.e.* open, hydroxy-acid) or inactive form (*i.e.* closed, lactone) (Figure 6C). Unlike synthetic statins, natural and semi-synthetic statins are obtained in lactone form, however, *in vivo*, these statins are enzymatically

hydrolyzed to the active form (Corsini et al., 1995). The natural and semi-synthetic statins all have a very similar structure (Figure 6); a core polyketide backbone, hexahydronaphtalene ring system with different side chains linked to C8 and C6. Lovastatin (also called mevinolin or monacolin K) differs from compactin only by the presence of methyl group attached to the C8. Simvastatin has an additional methyl group in the side chain, and pravastatin is a C6-hydroxy analogue of compactin. Besides the HMG-like moiety, synthetic statins do not share any other structural similarity to natural statins (Figure 6).

All statins are competitive inhibitors of HMGCR with respect to the binding of the substrate, HMG-CoA. With their HMG-like moiety, statins bind to the active site of HMGCR, thus sterically prevent the substrate from accessing and binding to the HMG-CoA binding site (Istvan and Deisenhofer, 2001).

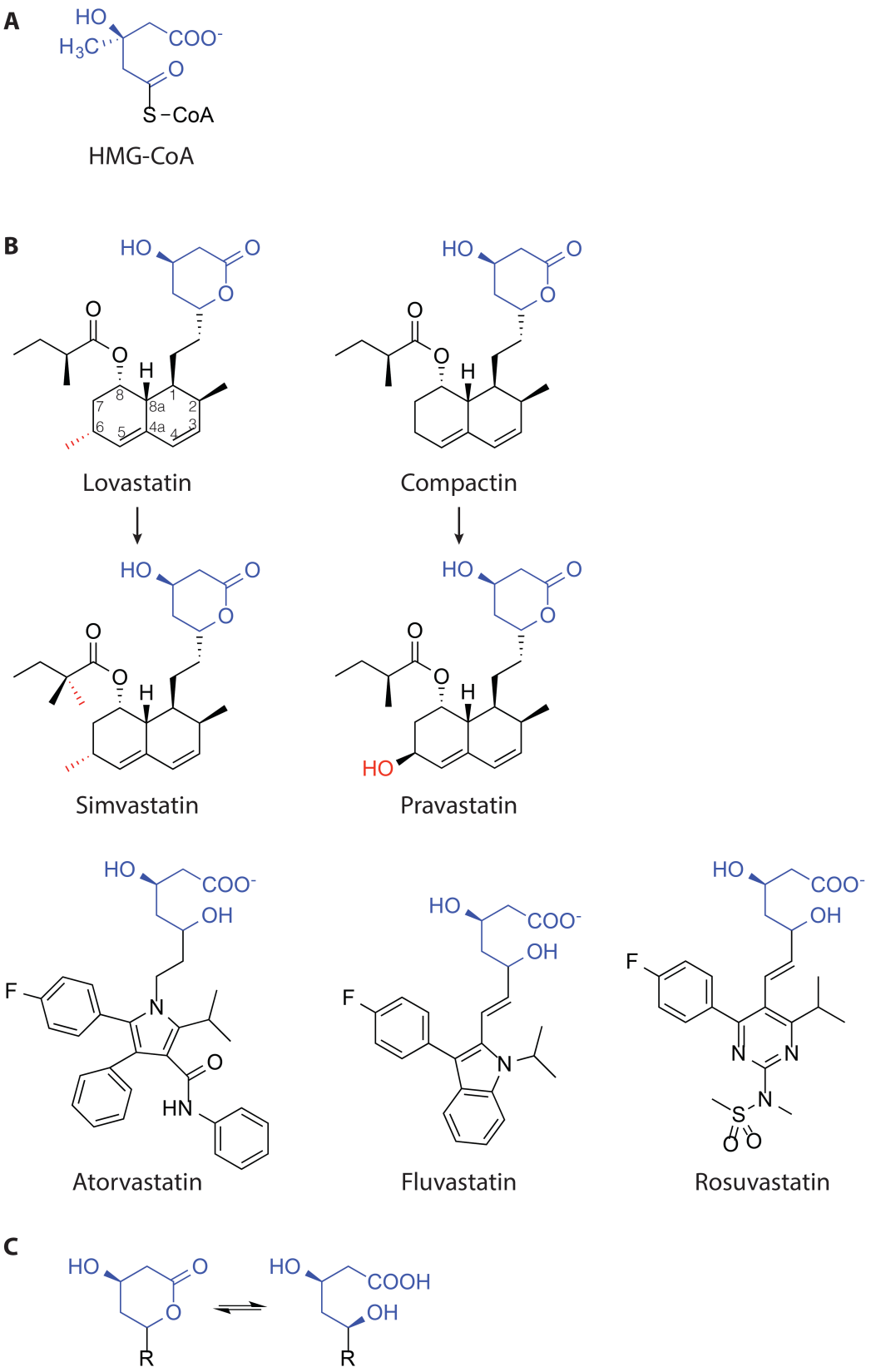

Figure 6: Structures of statins – HMGCR inhibitors – and the enzyme substrate HMG-CoA. (A) Structure of HMG-CoA. (B) Structures of natural statins (lovastatin and compactin), their semi-synthetic derivatives (simvastatin and pravastatin), and synthetic statins (atorvastatin, fluvastatin and rosuvastatin). (C) Statins exist in two forms: closed lactone form (left), and open hydroxyl acid form (right). The HMG-like moieties are colored blue; the unique groups of natural and semi-synthetic statins are colored red. Carbon atom numbering of the hexahydronaphtalene ring system of the natural and semi-synthetic statins is shown on the example of lovastatin.

2.3 Biosynthesis of natural statins

The natural statins are synthesized as secondary metabolites by many fungi. The first statin, i.e. compactin, was isolated from *Penicillium citrinum* (Endo et al., 1976a) in 1976, and shortly after, lovastatin was isolated from *Monascus ruber* (Endo et al., 1979) and *Aspergillus terreus* (Endo, 1979). Early biosynthesis studies on these fungi using labeled precursors revealed that both, compactin and lovastatin originate from a similar biosynthetic pathway, i.e. polyketide pathway starting with acetate (Endo et al., 1985; Kimura et al., 1990; Komagata et al., 1989; Moore et al., 1985). Moreover the biosynthetic gene clusters of lovastatin in *A. terreus* (Hendrickson et al., 1999; Hutchinson et al., 2000; Kennedy et al., 1999) and *M. pilosus* (Chen et al., 2008), and of compactin in *P. citrinum* (Abe et al., 2002b) all contain nine genes, encoding for enzymes with similar amino acid sequences (Figure 7). Most of the knowledge about the biosynthesis of the natural statins and the molecular genetics of the producer strains is based on the biochemical and genetic studies of the lovastatin biosynthetic pathway in *A. terreus*, I will therefore use lovastatin as an example to describe the statin biosynthesis in the next section.

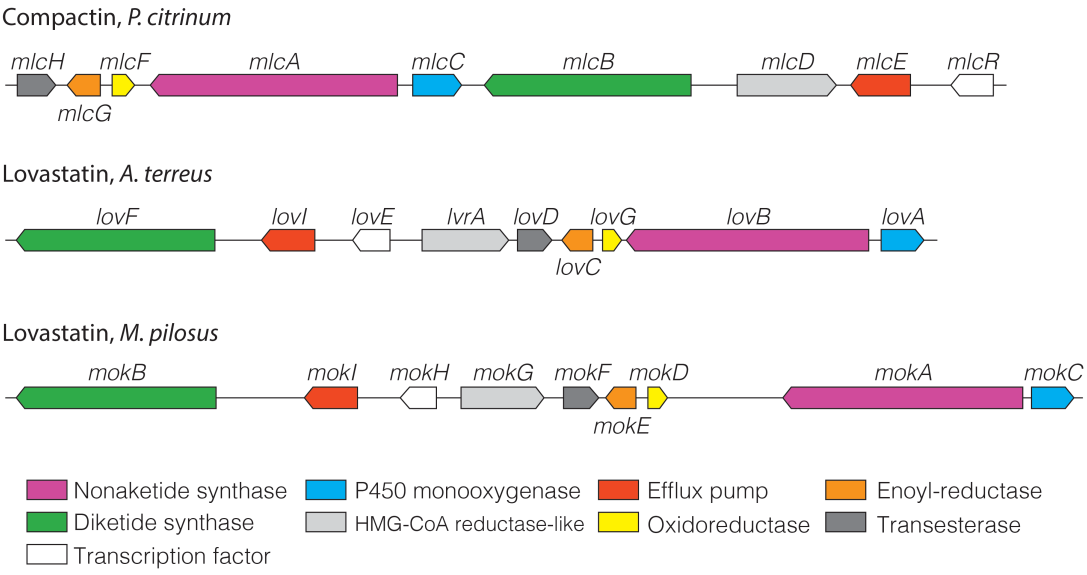

Figure 7: Biosynthetic gene clusters of natural statins in filamentous fungi.

2.3.1 Lovastatin biosynthesis

The polyketide backbone of lovastatin is synthesized by two highly reducing iterative type I polyketide synthases, LovB and LovF (Hendrickson et al., 1999; Kennedy et al., 1999). LovB contains seven active domains (Ma and Tang, 2007) (Figure 8) and is responsible for synthesizing of the first intermediate in the pathway, a nonaketide called dihydromonacolin L acid (Figure 9). The enzyme is hence called lovastatin nonaketide synthase. LovB catalyzes the Claisen condensation of nine malonyl-CoA units in successive iterations. After each condensation, the polyketide backbone is decorated by the use of different combinations of the tailoring domains, i.e. methytransferase (MT), ketoreductase (KR), and dehydratase (DH). As LovB contains an inactive enoyl reductase (ER) domain, the reduction of the nonaketide backbone is assisted by the trans-acting enoyl reductase LovC (Ames et al., 2012; Auclair et al., 2001; Kennedy et al., 1999), which specifically reduces tetraketide, pentaketide and heptaketide intermediates during the nonaketide synthesis.

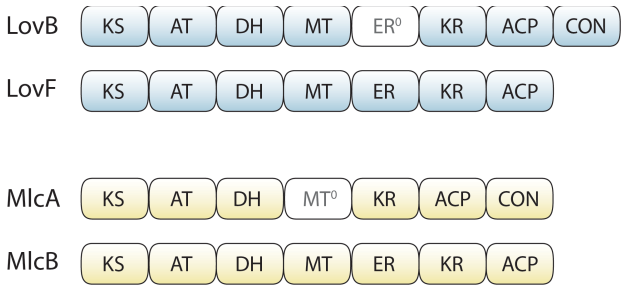

Figure 8: Domain structure of lovastatin and compactin polyketide synthases. LovB and LovF – Lovastatin nonaketide and diketide synthase, respectively. MlcA and MlcB – Compactin nonaketide and diketide synthase, respectively. KS – β-ketosynthase; AT – acyltransferase; DH – dehydratase; MT – methyltransferase (MT^0 – inactive); ER – enoyl reductase (ER^0 - inactive); KR – ketoreductase; ACP – acyl carrier protein; CON – condensation domain (adapted from (Campbell and Vederas, 2010)).

Besides the Claisen condensation, LovB is thought to have a Diels-Alderase activity responsible for formation of the fused rings of the decalin system of dihydromonacolin L (Auclair, 2000; Kelly, 2008; Witter and Vederas, 1996). Dihydromonacolin L acid is further modified (hydroxylation and dehydration) by the

cytochrome P450 monooxygenase LovA to produce monacolin L acid, which is further hydrolyzed by LovA to afford monacolin J acid (Figure 9) (Barriuso et al., 2011). Lovastatin diketide synthase LovF differs from LovB by the presence of an active ER domain (Figure 8) and is responsible for synthesizing the α-methylbutyryl side chain of lovastatin by condensation and reduction of two acetyl units (Kennedy et al., 1999). The LovF formed diketide chain is transferred to the C-8 hydroxy group of monacolin J acid by the acyl transferase LovD (Xie et al., 2009), resulting in the formation of lovastatin acid (Figure 9). One unique feature of highly reducing polyketide synthases, also found in the two lovastatin polyketide synthases, LovB and LovF, is the lack of a functional thioesterase (TE) domain, which in other types of polyketide synthases releases the product form the enzyme (M. Wang et al., 2009; Zhou et al., 2010a). *In vitro* biochemical studies have revealed that the acyl transferase LovD removes the α-methylbutyryl side chain from LovF via direct protein-protein interaction and transfers it to monacolin J acid, thereby freeing the active site of LovF allowing for initiation of a new synthesis (Kennedy et al., 1999; Xie et al., 2009). Similarly, LovB is dependent on the activity of stand-alone thioesterase LovG, which releases the formed dihydromonacolin L acid from LovB (Xu et al., 2013). Furthermore, LovG is also responsible for the clearance of irregular intermediates from LovB (Xu et al., 2013).

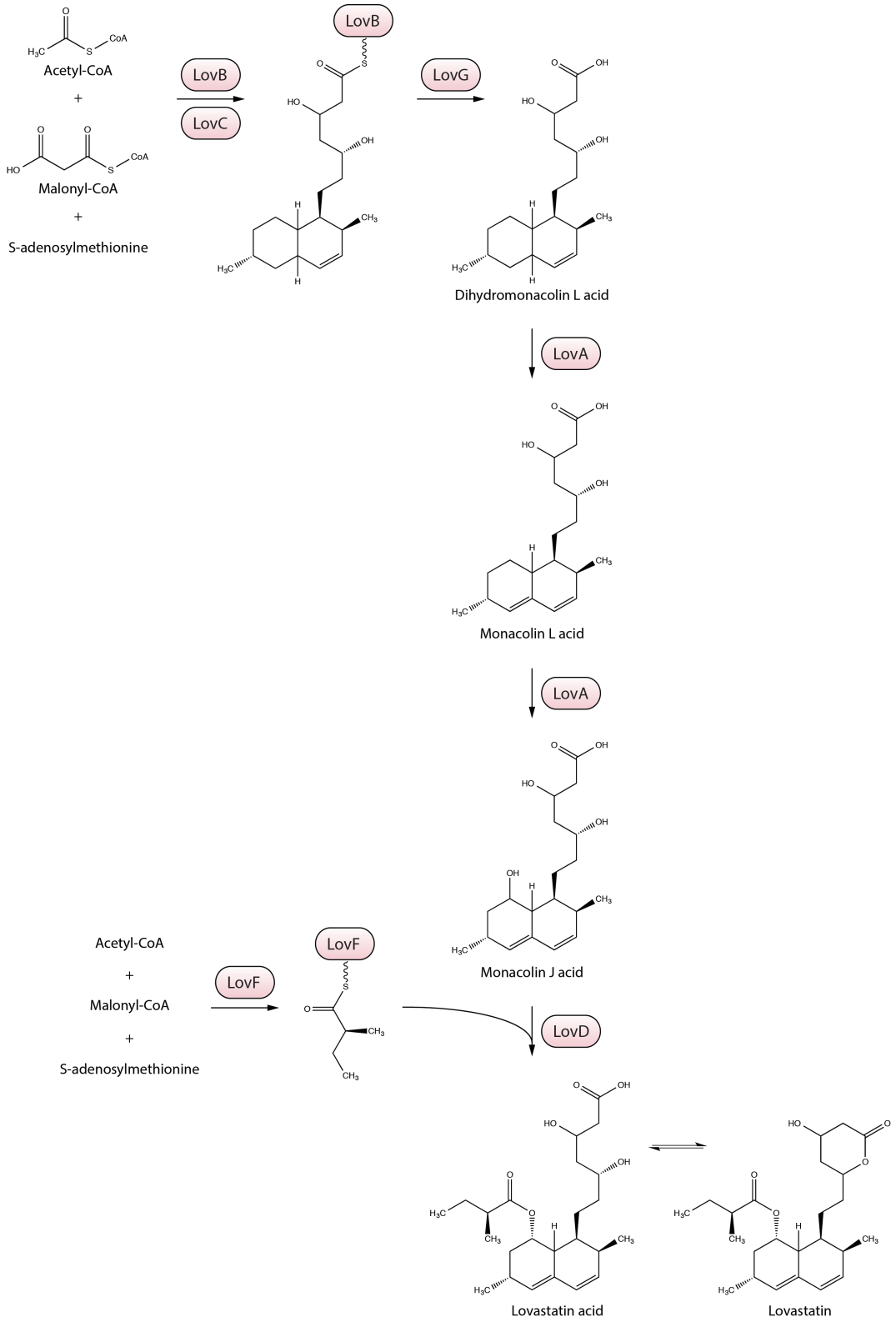

Figure 9: Lovastatin biosynthetic pathway. LovB (lovastatin nonaketide synthase) produces the first intermediate Dihydromonacolin L acid, which is released form the enzyme by the stand-alone thioesterase LovG. The nonaketide backbone is tailored by enoyl reductase LovC and cytochrome P450 monooxygenase LovA. The diketide portion of lovastatin is synthesized by LovF (lovastatin diketide synthase), which is transferred to the nonaketide intermediate monacolin J acid by acyl transferase LovD (adapted from (Ma et al., 2009a; Xu et al., 2013)).

2.3.2 Compactin biosynthesis

The structure of compactin is very similar to lovastatin, and so is the biosynthetic pathway and the enzymes involved in the biosynthesis (Abe et al., 2002b). The compactin nonaketide synthase MlcA, like LovB, requires a trans-acting enoyl reductase, in this case MlcC, to synthesize the first nonaketide intermediate, due to the lack of the thioesterase domain. The methytransferase (MT) domains present in LovB and LovF were also found in MlcA and the compactin diketide synthase MlcB. The methyl group, which is introduced at the C6 position of lovastatin is not present in the compactin structure, therefore it is thought that the MT domain in MlcA is inactive (Abe et al., 2002b).

Transcription of the statin biosynthetic genes is regulated by the transcriptional activators (*mlcR* and *lovE*), which are also encoded in the statin gene clusters (Figure 7) (Baba et al., 2006; Chen et al., 2010; Hutchinson et al., 2000). Synthesis of many secondary metabolite gene clusters in filamentous fungi are controlled by global regulators, such as LaeA and VeA (Bayram et al., 2008; Bok and Keller, 2004; Keller et al., 2005), and it has been shown that the two regulators also control the compactin synthesis in *P. citrinum* by modulating the expression of *mlcR*, a transcriptional activator from the compactin cluster (Baba et al., 2012).

2.4 Biotechnological production of natural and semi-synthetic statins

Although most of the newer statins are of synthetic origin, the microbial statins and their semi-synthetic derivatives are still the first-line treatment of choice for patients with high plasma cholesterol and CHD (dos Santos et al., 2014; Tobert, 2003), simvastatin being the third most prescribed drug in the United States (IMS, 2012).

Fermentation-based processes using filamentous fungi are the core technology in the production of natural and semi-synthetic statin. Lovastatin is produced by a variety of filamentous fungi, among which different *Monascus* (Endo, 1979; Miyake et al., 2006; Sayyad et al., 2007; Seraman et al., 2010) and *Aspergillus* (Alberts et al., 1980; Benedetti et al., 2002; Casas López et al., 2003; Gupta et al., 2007; Kumar et al., 2000; Porcel Rodriguez et al., 2008; Samiee et al., 2003) species were found to be the most significant producers, however, the commercial production of lovastatin is mostly based on the fermentation of *Aspergillus terreus*, and the majority of the research towards improved production of statins has been based on this species. Compactin is produced by several species of *Penicillium* (Brown et al., 1976; Choi et al., 2004; Endo et al., 1976a, 1976b; ZafferAhamad et al., 2006), and although it is not used medically due to its many side effects, it represents an important source for the production of pravastatin, a more effective statin. Most of the biotechnological production processes of natural statins are based on the liquid submerged fermentation, however in the past decade the solid state fermentation (SSF) became a promising alternative production system as it showed to have several advantages for the production of statins, e.g. higher product yield, lower energy requirement etc. (Baños et al., 2009; Mahesh et al., 2012; Praveen and Savitha, 2012; Raghavarao et al., 2003; S. Singh and Pandey, 2013).

Compactin is converted to pravastatin in a second stage fermentation via biotransformation (hydroxylation) (Endo et al., 1979; Terahara and Tanaka, 1982) by different bacteria; *Streptomyces* (Gururaja et al., 2003; Matsuoka et al., 1989), *Nocardia* (Serizawa et al., 1983a, 1983b), *Actinomadura* (Peng et al., 1997; Yashphe et al., 1997). One of the major problems during the biotransformation of compactin to pravastatin is the sensitivity of bacteria to compactin, therefore, searching for the conversion organism must include screening for the compactin resistance (Chen et al., 2006). Two-step fermentation requires purification of compactin and its subsequent biotransformation in another fermentation process, reducing the yields

and increasing the cost of the compound (Hosobuchi et al., 1993; Park et al., 2003). Therefore, efforts towards developing one-step fermentation process have been made; a hydrolase gene from *Streptomyces carbophilus* that is usually employed in the second stage fermentation, has been inserted into the compactin producing *P. citrinum*, and the transformation resulted in a pravastatin producing strain (Luiten et al., 2000). Recently, introduction of the compactin biosynthetic pathway and *Amycolatopsis orientalis* cytochrome P450 into *Penicillium chrysogenum* resulted in a successful single-step fermentative production of pravastatin (McLean et al., 2015).

Simvastatin is traditionally obtained via a semi-synthetic multistep process, where the 2-methylbutyrate side chain of lovastatin is chemically modified to 2,2-dimethylbutyrate (Askin et al., 1991; Hoffman et al., 1986). As the semi-synthetic conversion is very laborious, simvastatin is approximately five times more expensive than lovastatin (Barrios-González and Miranda, 2010), and numerous efforts have been made towards establishing a biotechnological process for simvastatin production. A one-step whole-cell biocatalytic process has been established by Xie et al (Xie and Tang, 2007) by expressing acyltransferase LovD in *Escehrichia coli*, which was able to convert monacolin J to simvastatin. Moreover, the broad substrate specificity of LovD (Xie et al., 2006) was exploited, and a suitable membrane-permeable acyl donor (α-dimethylbutyryl-S-methyl-mercaptopropionate (DMB-SMMP)) was found to be efficiently utilized by LovD in this process (Xie and Tang, 2007). To further improve simvastatin production in this biocatalytic process LovD has been engineered using directed evolution (Jiménez-Osés et al., 2014). The activity of wild-type LovD in *A. terreus* is depended on acyl-carrier protein (ACP) domain of LovF, and has a poor activity towards the non-natural donor DMB-SMMP, thus Jimenez-Oses et al engineered a LovD, which does not require the ACP, and accepts a free DMB-SMMP, making it significantly more efficient for the simvastatin synthesis (Jiménez-Osés et al., 2014).

The high impact of statins to human health and their commercial value are driving research towards improved biotechnological production of statins. Besides the optimization of the existing production processes and statin-producing fungal strains, production of statins in a heterologous host represents an attractive approach, which remained largely unexploited. The possibility to synthesize statins in a heterologous host is discussed in Chapter 4.

CHAPTER 3

Self-resistance to secondary metabolites

3.1 Introduction

Secondary metabolites (e.g. antibiotics and mycotoxins) are often toxic to the cells that synthesize them; therefore a specific self-resistance mechanism must be present in these cells to avoid self-intoxication (Cundliffe, 1989; Demain, 1974). Most secondary-metabolite producing organisms carry genes for the self-protection, and these genes are usually found in clusters together with the genes encoding for the biosynthetic machinery and regulation (Hopwood, 2007; Martín et al., 2005), as described in Chapter 1. Very little is known about self-resistance mechanisms in fungi, which is surprising considering the impact that fungal secondary metabolites have in human health. This is also the case for industrially relevant systems such as the statin-producing filamentous fungi. Elucidation of the self-resistance mechanism to statins is one of the goals of this project; therefore I will introduce the concept of self-resistance in the present chapter.

The self-toxicity of secondary metabolites is not an issue only in fungi but also in plants and bacteria, where much more knowledge regarding the self-protection mechanisms is available. Insight into the self-resistance mechanisms in one type of organisms can hopefully guide discovery and understanding of similar or alternative solutions to the same basic problem in other types of organisms. In this chapter I will provide an overview of known self-resistance mechanisms to secondary metabolites including examples from both, plants and microorganisms (bacteria and fungi). Organisms respond to toxic compounds in many different

ways, however some mechanisms are compound-specific, and some are of a general nature, responding to a variety of different compounds, and some are compound-specific responses. The focus in this chapter will be on mechanisms, for which the encoding genes are found within the secondary metabolite gene clusters, as these are presumed to be metabolite-specific mechanisms.

Mechanisms by which producers can overcome the deleterious effects of producing a bioactive compound have been studied extensively in the antibiotic-producing bacteria, and mechanisms utilized by plants and fungi have typically been identified in connection with studies of plant diseases (Coleman and Mylonakis, 2009; Del Sorbo et al., 2000; Sirikantaramas et al., 2008). The main mechanisms of self-resistance are common in all three types of organisms, and rely on the reduction of the metabolite's concentration at the site where it could have a toxic effect. This can be achieved by secreting the secondary metabolite from the cell by an efficient transport system (Figure 10, part 1), modifying the metabolite into an inactive or less active form (Figure 10, part 2), or sequestering the metabolite into compartments where the target is not present (Figure 10, part 3). Moreover, the synthesis of the metabolite itself can be controlled by a negative feedback mechanism that responds to the increased concentrations of the metabolite in the cell, and thereby prevents further increases in the compound concentration (Figure 10, part 4). Another concept used to confer self-resistance is based on prevention of the target-metabolite interaction by alteration of the target site. This can be achieved by posttranslational modification of the target, or synthesis of an insensitive version of the target (Figure 10, parts 5 and 6, respectively). The target protein can also be overexpressed resulting in reduction of the relative concentration ratio between the bioactive compound and the target protein (Figure 10, part 7). In the following sections the different mechanisms are described and examples from all three types of organisms (bacteria, plants, fungi) are provided.

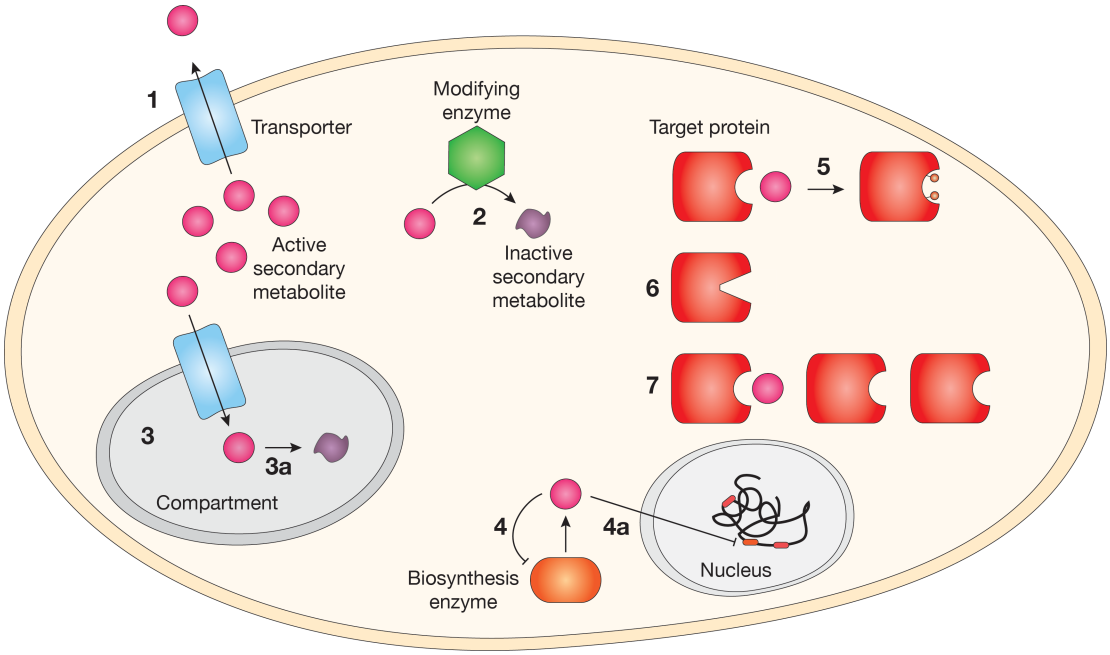

Figure 10: Mechanisms of self-resistance to secondary metabolites. (1) Secretion, (2 and 3a) modification, and (3) sequestration of the metabolite. (4 and 4a) Feedback inhibition of metabolite biosynthesis. Target alteration by (5) posttranslational modification of the target, (6) synthesis of an insensitive version of the target, and (7) target overexpression.

3.2 Metabolite secretion by transport proteins

The transport proteins encoded by genes located in the secondary metabolite gene clusters can serve several purposes. Firstly, they ensure that the endogenously produced secondary metabolites reach the extracellular environment, where they can act as communication signals, alter the local environment in a way to fit the needs of the producing organisms, inhibit the growth of or kill other organisms (Martín et al., 2005). Secondly, an efficient efflux mechanism of the metabolites protects the producing cells from self-intoxication (Coleman and Mylonakis, 2009; Cundliffe, 1989; Del Sorbo et al., 2000). Transport proteins are also involved in movement of the intermediates between the different cellular compartments. The transporters found in the secondary metabolite gene clusters are structurally diverse, and can be divided into two major classes based on the

source of energy they are using; (i) ATP-binding cassette (ABC) transporters, and (ii) major facilitator superfamily (MFS) transporters (Figure 11).

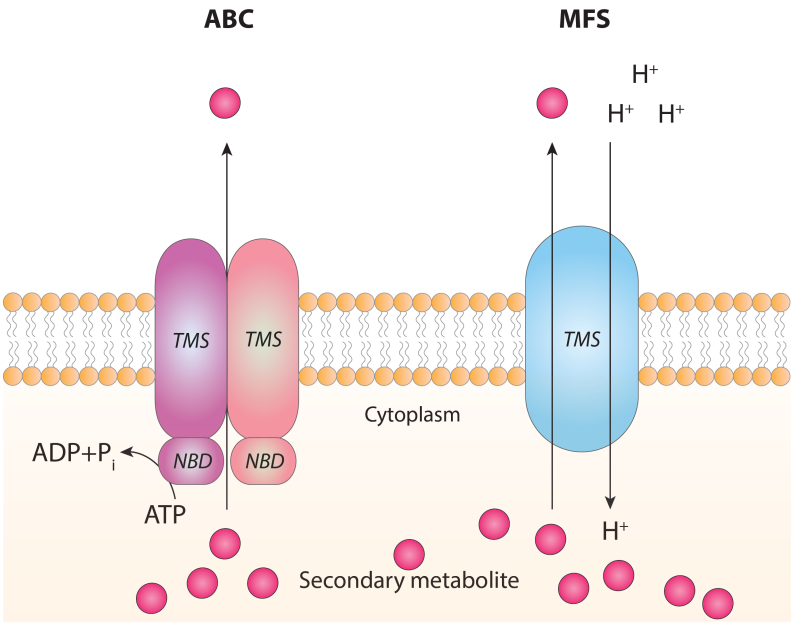

Figure 11: Main classes of transporters involved in the secondary metabolite secretion and self-resistance; ABC transporters hydrolyze ATP in order to secrete the metabolites and are usually composed of two nucleotide binding domains (NBD), and two hydrophobic regions, each containing 6 transmembrane domains (TMS). MFS transporters use the transmembrane electrochemical gradient of protons to transport the metabolites across the membrane and contain 12 or 14 TMS (adapted from (Coleman and Mylonakis, 2009; Piddock, 2006).

ABC transporters use the energy from ATP hydrolysis to export the metabolite. Most ABC transporters are composed of nucleotide-binding domain (NBD), followed by a hydrophobic region containing 6 transmembrane domains (TMS). The two parts can be synthesized as one polypeptide, which dimerize to form a fully functional protein, however, in fungi a single polypeptide containing two NBD and two TMS regions is synthesized (Higgins, 1992; Hyde et al., 1990; Saier, 2003). Numerous ABC transporters from bacterial secondary metabolite gene clusters have been characterized, mostly from different antibiotic-producing *Streptomyces* species (Fernández et al., 1996; Linton, 1994; Olano, 1995; Rosteck, 1991; Schoner et al., 1992). In contrast, only a few examples of secondary-metabolite ABC transporters have been found in fungal secondary metabolite gene clusters; SirA confers self-resistance to sirodesmin in *Leptosphaeria maculans*.

Sirodesmin is a cyclic dipeptide with a disulfide bridge that generates reactive oxygen species (ROS) in the cells and interacts with susceptible thiol residues on proteins, resulting in their inactivation. *L. maculans sirA* mutant was considerably more sensitive to sirodesmin than the wild-type strain (Gardiner et al., 2005). FUM19 from *Fusarium verticillioide* was proposed to confer the self-resistance to fumonisin - inhibitor of ceramide synthase, enzyme involved in sphingolipid metabolism, because the gene encoding for this transporter is located in the fumonisin biosynthetic gene cluster and its deletion affected the synthesis of fumonisin, however, the direct role in self-resistance is yet to be confirmed (Proctor et al., 2003). Other ABC transporters found to be conferring self-resistance to secondary metabolites in fungi are ATR1, efflux pump for ROS-generating compound cercosporin from *Cercospora nicotianae* (Amnuaykanjanasin and Daub, 2009), and AtrD transporter from *Aspergillus nidulans* capable of secreting penicillin (Andrade et al., 2000). Special for the two latter examples is that the genes encoding the pumps are located outside the gene clusters encoding the biosynthetic apparatuses. ABC transporters are also important in conferring the self-resistance in plants (Jasiński et al., 2001; Stukkens et al., 2005; Van Den Brûle et al., 2002).

Members of MFS of transporters are characterized by the presence of a single polypeptide that usually includes 12 or 14 TMS. They are capable of transporting only small molecules and utilize the transmembrane electrochemical gradient of protons to drive the transport of the metabolites (Pao et al., 1998; Paulsen et al., 1996). Like ABC transporters, most of the characterized MFS transporters originate from *Streptomyces spp.*, where they typically are responsible for exporting antibiotics, such as cephamycins (Coque, 1993; Liras, 1999; Pérez-Llarena et al., 1998) and tetracycline (Ohnuki et al., 1985; Reynes et al., 1988). In fungi, MFS transporters are the most numerous types of transporters (Coleman and Mylonakis, 2009). Despite their abundance, only a few MFS transporters have been functionally characterized, and shown to be directly involved in self-resistance to secondary metabolites. GliA, MFS transporter from *Aspergillus fumigatus* encoded

in the gliotoxin biosynthetic gene cluster, exports this ROS-generating compound from the fungus (Wang et al., 2014). Similarly, TOXA exports HC-toxin, which is an inhibitor of histone deacetylases, from *Cochliobolus carbonum* (Pitkin et al., 1996), TRI12 exports trichothecene toxin, an inhibitor of protein synthesis, from *Fusarium sporotrichioides* (Alexander et al., 1999), and CFP is capable of secreting cercosporin from *Cercospora kikuchii* (Callahan et al., 1999; Upchurch et al., 2002).

Secondary metabolite gene clusters containing more than one transporter have also been described. The gene encoding for a HC-toxin efflux pump *TOXA* is present as two linked copies in most toxin-producing isolates (Pitkin et al., 1996). Moreover, the cephalosporin cluster in *Acremonium chrysohenum* contains two genes encoding for the putative MFS transporters, *cefM* and *cefT*, and a gene encoding for a transporter of an uncharacterized family, *cefP*. While CefT exports β-lactam antibiotic cephalosporin from the cells and likely confers self-resistance (Ullán et al., 2002), CefM and CefP translocate the intermediates between cellular compartments, and are thereby directly involved in the cephalosporin biosynthesis. CefP transports isopenicillin N into peroxisomes (Ullán et al., 2010), where it is converted to penicillin N. The latter is then transported from the peroxisomes to the cytosol by CefM, where it is converted into the final compound cephalosporin C (Teijeira et al., 2009). This example shows that transport proteins also play an important role in the intracellular transport of secondary metabolites between the different compartments, a process that can also confer the self-resistance, as described in the next section. The role of transporter proteins in sequestrations is well known in plants, where certain ABC transporters transfer secondary metabolites into vacuoles (Goodman et al., 2004; Klein et al., 2000; Lu et al., 1997).

3.3 Sequestration of the metabolite by compartmentalization

Spatial organization is one of the least studied aspects of secondary metabolism. Nevertheless, studies of both myco- and phytotoxin biosynthetic pathways in the past decade have revealed that the key elements in secondary metabolism (i.e. biosynthetic enzymes, intermediates and end products) are often localized in distinct sub-cellular compartments (vacuoles, peroxisomes, vesicles, cytoplasm), in highly specialized cells, or in different tissues. One possible role of the compartmentalization of secondary metabolites is that sequestration can provide self-resistance (Roze et al., 2011; Sirikantaramas et al., 2008). This type of self-resistance is often found in plants. In many cases the toxic metabolites are transported into the vacuole, where they can accumulate without damaging the cells, a common mechanism of self-protection against flavonoids (e.g. anthocyanins (Ahmed, 1994; Rueff, 1995)) and alkaloids (Deus-Neumann and Zenk, 1984). In addition to the compartmentalization of the toxic compounds, the synthesis of the metabolites can be carried out in specialized compartments, as in the synthesis of cannabinoids in glandular trichomes (Sirikantaramas et al., 2005), compartments that are essentially outside of the plant body (Wagner, 1991). Different vesicles are also involved in the secondary metabolite biosynthesis and self-resistance in fungi. Penicillin biosynthesis in *Penicillium chrysogenum* is compartmentalized in Golgi-derived vesicles, cytosol and peroxisomes (Kuryłowicz et al., 1987; Meijer et al., 2010; Van De Kamp, 1999), however localization of the first catalytic enzyme in the synthesis (ACVS[3]) is still controversial (Lendenfeld et al., 1993; Müller et al., 1991; van der Lende et al., 2002). Similarly, the aflatoxin biosynthesis in *Aspergillus parasiticus* is carried out in several different vesicles, including specialized vesicles named aflatoxisomes, which are thought to confer the self-resistance to the mutagenic aflatoxin (Figure 12) (Chanda et al., 2009; Hong and Linz, 2009, 2008;

[3] δ-(L-α-amino- adipyl)-L-cysteinyl-D-valine (ACV) synthetase

Lee et al., 2004). *Tolypocladium inflatum* has been shown to deposits cyclosporin in the vacuole upon its complete synthesis (Hoppert et al., 2001). Besides the self-protection role, the different vesicles in fungi are predicted to have a role in storage, protein turnover, transport or export of the secondary-metabolites by exocytosis as an alternative secretion mechanism to the transporter-mediated export. Compartmentalization can also ensure a high concentration of substrates, cofactors and intermediates at the same site where key enzymes are present, thus increase the flow through the pathway, and can prevent cross-chemistry between the pathways (Chanda et al., 2009; Roze et al., 2011). Specific conditions in a compartment, such as the low pH of the vacuole, can theoretically also modify the compound resulting in the inactivation of the compound (Figure 10, part 3a).

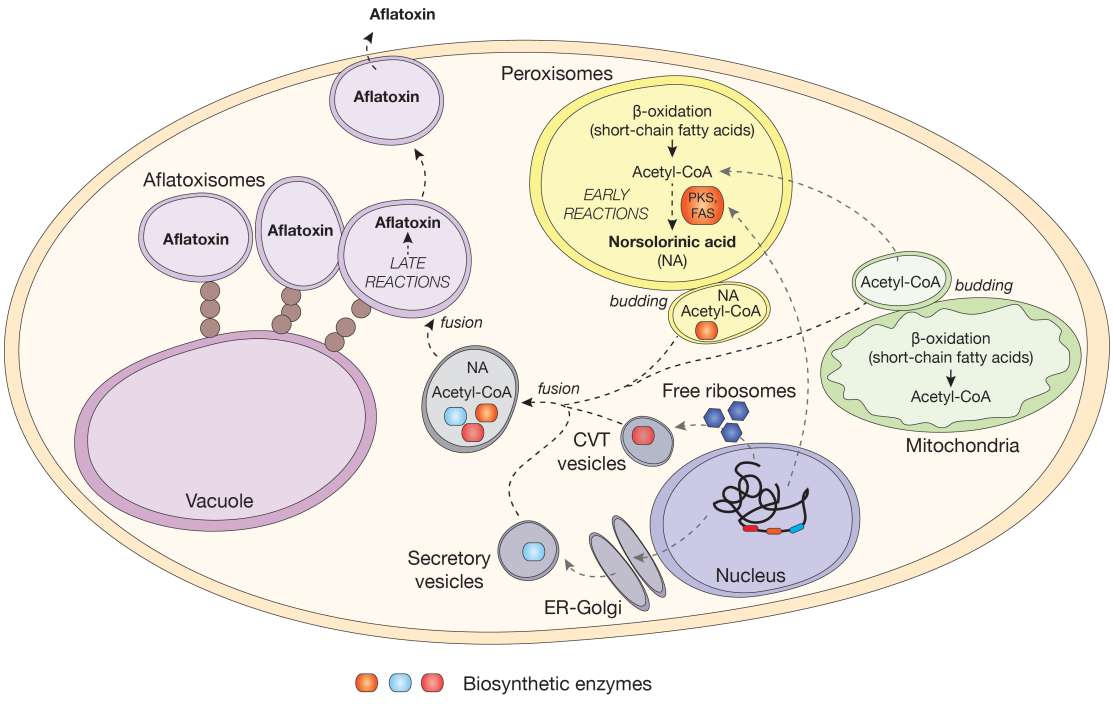

Figure 12: Model for compartmentalization of the aflatoxin biosynthesis in *Aspergillus spp*. The first steps in aflatoxin biosynthesis are carried out in peroxisomes. It is not known how the early enzymes (PKS, FAS) translocate into the peroxisomes. Vesicles originating from peroxisomes and mitochondria fuse with CVT (cytoplasm-to-vacuole-transport) vesicles that contain middle and late aflatoxin enzymes, and eventually with secretory vesicles to develop aflatoxisomes. The late stages in aflatoxin biosynthesis are carried out in aflatoxisomes, which are also responsible for the aflatoxin export (adapted from (Chanda et al., 2009; Kistler and Broz, 2015; Roze et al., 2011)).

3.4 Enzymatic metabolite modification

Several secondary-metabolite producers possess enzymatic systems capable of modifying endogenously produced metabolites, transforming them into less active or inactive forms (Figure 10, part 2). These modifications need to be reversible in order to keep the ability of the compounds to play their role in the organisms' environment. Enzymatic metabolite modification is a common mechanism of self-resistance in antibiotic-producing bacteria, where – despite the chemical diversity of the produced antibiotics – two modes of metabolite modifications are prevailing; N-acetylation of amino groups and O-phosphorylation of hydroxyls (reviewed by (Cundliffe, 1989)). Reduction and glycosylation of antibiotics in some *Streptomyces spp.* have also been identified as mechanisms of self-resistance (Cundliffe, 1992; Jenkins and Cundliffe, 1991; Lee et al., 1996; Zhao, 1998). In plants, glycosylation is an important detoxifying mechanism and several toxic metabolites have been shown to be stored as glucosides, e.g. benzoxazinoids (Osbourn, 1996; Schulz and Wieland, 1999; Sicker et al., 2000), reactive phenylpropanoids (Meyermans et al., 2000), cyanogenic glucosides (Conn, 1980) and glucosinolates (Rask et al., 2000). In fungi, only one example of metabolite modification as a self-protection mechanism have been published so far; GliT is an oxidase encoded in the gliotoxin biosynthetic gene cluster of *Aspergillus fumigatus*, responsible for oxidation of gliotoxin and thereby formation of the disulfide bridge in this compound. In addition to its role in the biosynthesis, GliT has also been shown to confer the self-resistance to gliotoxin in this fungus, probably by preventing unwanted redox cycling, as well as conjugation of gliotoxin with susceptible proteins. It has also been proposed that, since reduced gliotoxin was not found in the broth of the GliT-deletion mutant, the oxidized form with the disulfide bridge might be the requirement for the gliotoxin export (Scharf et al., 2010). Reduction of cercosporin was proposed to provide the self-resistance to this toxic compound in

Cercospora spp., but it is not known how does the reduction take place (Daub et al., 1992).

3.5 Feedback regulation

Biosynthesis of secondary metabolites is often subjected to negative feedback regulation. Because primary and secondary metabolism share a pool of precursors, it has been proposed that antibiotic producers evolved negative feedback inhibition of the secondary metabolite's synthesis to ensure the maintenance of the primary metabolism (Koehn, 2013). However, since secondary metabolites can be toxic to the producers, one might consider the feedback regulation as a way of self-protection. The exact mechanism of feedback regulation is often not known, but some of the examples suggest that the secondary metabolite can either directly inhibit the activity of a preexisting biosynthetic enzyme (Figure 10, part 4) (Hamano et al., 2007), or it can affect expression of the genes involved in its biosynthesis and regulation, a mechanism also referred to as feedback repression (Sanchez and Demain, 2002) (Figure 10, part 4a) (Ajithkumar and Prasad, 2010; Clay et al., 2009; Srinivasan et al., 2010). As described in previous sections, transporters often maintain subinhibitory physiological concentration of the secondary metabolites within the cells. Deletion of these transporters in the secondary metabolite producing cells can result in increased sensitivity of the cells to toxic metabolites added externally in the medium, but it can also lead to the decreased production of the compound. This has been shown for the cercosporin and trichothecene specific efflux pumps, where deletion of the encoding genes reduced the synthesis of the given secondary metabolite in fungus (Alexander et al., 1999; Amnuaykanjanasin and Daub, 2009; Callahan et al., 1999; Choquer et al., 2007). Results, which suggest that a negative product feedback regulation exist in these systems, and that the pumps prevent the intracellular accumulation of the product. Regulatory feedback mechanism is also present in the

lovastatin biosynthesis in *A. terreus*, however it is not clear how does lovastatin inhibits its own synthesis (Casas López et al., 2004).

3.6 Target alteration

Targets of bioactive secondary metabolites are often enzymes involved in the primary metabolism or in the synthesis of macromolecules, and self-resistance mechanisms involving the target enzymes exist. Posttranslational modification of a sensitive target enzyme, which weakens or prevent the interaction of the enzyme with the secondary metabolite, can afford self-resistance to the given compound (Figure 10, part 5). Self-resistance can be also achieved by replacement of the target enzyme with its resistant version; in some cases a resistant enzyme have evolved with altered amino acid sequence that prevents the interaction of the enzyme with the toxic compound (Figure 10, part 6). Such resistant enzyme is co-expressed with the genes encoding for the biosynthetic pathway. Alternatively, the target enzyme might be overexpressed (Figure 10, part 7), resulting in sufficient activity in the presence of the toxic secondary metabolite. The basic concept behind the latter mechanism is reduction of the relative concentration ration between the bioactive compound and the target protein; nevertheless, the mechanism involves the target enzyme, and is therefore included in this section.

Resistance enzymes that modify the metabolite target site are common in bacteria that produce inhibitors of protein synthesis; *Streptomyces spp.* synthesizing thiostrepton (Thompson et al., 1982), kanamycin (Nakano et al., 1984) or erythromycin (Skinner and Cundliffe, 1982; Teraoka and Tanaka, 1974) have ribosomal RNA (rRNA) methylases that confer the self-resistance by introducing a single methyl group into rRNA, an alteration that prevents the antibiotics to bind to this target molecule, the ribosome. As for many other self-resistance mechanisms, the encoding genes for rRNA methylation are located within the antibiotic biosynthesis gene clusters (Stanzak et al., 1986; Yanai and Murakami, 2004).

Many examples of the synthesis of resistant version of the target protein have been described in bacteria that produce antibiotics with non-ribosomal targets. Novobiocin is an inhibitor of DNA gyrase, and the producer of this antibiotic *Streptomyces sphaeroides* carries two genes encoding for DNA gyrase in its genome. One of these two genes is located in the novobiocin biosynthetic gene cluster (Steffensky et al., 2000) and its expression is induced by novobiocin. The DNA gyrase encoded within the cluster is resistant to novobiocin, whereas the product of the second gene, which is constitutively expressed, is novobiocin-sensitive (Thiara and Cundliffe, 1989, 1988). Another example was shown in the pentalenolactone (PL)-producing *Streptomyces spp*. PL inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the producer contains two GAPDH isozymes, one sensitive to PL, and one PL-insensitive. Gene encoding for the PL–insensitive GAPDH is located in the PL biosynthetic gene cluster, and its transcription is increased upon the PL synthesis (Fröhlich et al., 1989; Tetzlaff et al., 2006). Kirromycin produced by *Streptomyces ramocissimus* inhibits protein synthesis by binding to the polypeptide chain elongation factor (EF)-Tu (Olsthoorn-Tieleman et al., 2007). Gene encoding for a kirromycin-resistant EF-Tu has been found in *Streptomyces ramocissimus*, however, kirromycin does not induce transcription of this putative self-resistance gene (Glöckner and Wolf, 1984). Interestingly, the producing organism contains two more genes encoding for EF-Tu, which are constitutively expressed and the product of both is kirromycin sensitive (Olsthoorn-Tieleman et al., 2007; Vijgenboom et al., 1994), therefore overexpression of the target enzyme might be the mechanism of self-resistance in this case. Only two examples of target alteration as a self-resistance mechanism in fungi have been published so far. Camptothecin producing fungus *Fusarium solani* protects itself from this DNA topoisomerase I inhibitor by synthesizing DNA topoisomerase I with a structure that prevents camptothecin from binding to it (Kusari et al., 2011), however the location of the self-resistance gene in the fungal genome is not known. A resistant version of inosine-5-monophosphate (IMP) dehydrogenase, a target of

mycophenolic acid (MPA) was found in the MPA producer *Penicillium brevicompactum*, and the gene encoding for this enzyme lies within the MPA biosynthetic cluster (Hansen et al., 2011; Sun et al., 2011).

3.7 Self-resistance is a complex system

From the examples above it is clear that one self-resistance mechanism can serve multiple different functions. Compartmentalization of secondary metabolites function as sequestration, but it can also play a role in the modification/activity of the metabolites, as specific conditions in the compartment (e.g. low pH in the vacuole) can affect the structure, and thereby activity of the compound. This duality is similar for efflux pumps, which secrete the secondary metabolites out of the producing cells, but are also involved in metabolite sequestration by transporting them into different compartments. Moreover, secondary metabolite biosynthetic gene clusters, in both bacteria and fungi, often include more than one gene encoding for self-resistance mechanism, one of them being a transporter (either MFS or ABC) in most of the published examples (Pernodet et al., 1993; Proctor et al., 2003; Siegers and Entian, 1995; Wang et al., 2014). Thus, self-resistance is typically not a single-protein activity, but rather a complex system composed of several mechanisms, which allows for the production of increased concentrations of the bioactive compound.

3.8 Engineering self-resistance for improved biotechnological processes

Understanding the molecular biological basis underlying the self-resistance mechanisms in secondary metabolite producers can provide novel tools for improvement of biotechnological processes. Especially knowledge regarding the molecular mechanisms of metabolite secretion has an important, but so far largely

ignored, biotechnological potential as it can be used for strain engineering to increase the secretion of these product metabolites reducing purification costs. In addition to the improved resistance and reduced purification cost, the increased secretion of the products will also remove the negative feedback inhibition that sometimes limits the biosynthesis of the end product. Up to 2-fold increase in the cephalosporin production, a β-lactam antibiotic from fungus *Acremonium chrysogenum*, was observed when the MFS transporter gene *cefT* was overexpressed (Ullán et al., 2002). Moreover, overexpression of ABC transporters improved production of several medically important compounds; the productivity of antibiotic avermectin in *Streptomyces avermitilis* was 1.5 times higher upon overexpression of the AvtAB transporter (Zhang et al., 2002b). Similarly, an engineered strain of *Streptomyces peucetius* expressing multiple copies of ABC transporter DrrAB enhanced the production (2.2-fold increase) of an antitumor compound doxorubicin and had a longer life span than the parental strain (Malla et al., 2010). Production of doxorubicin in *Streptomyces peucetius* was also improved (5.1-fold increase) when another self-resistance gene was overexpressed, *drrC* encoding for an UvrA-like protein that protects the cells through excisional repair of DNA (Lomovskaya et al., 1996; Malla et al., 2010). In *Streptomyces coelicolor* it has been shown that the expression of ABC transporter ActAB is a critical determinant of antibiotic actinorhodin yields. Deletion of the transcriptional repressor of *actAB* operon resulted in a 4- to 5-fold increase in actinorhodin production (Xu et al., 2012).

In cases where secondary metabolites inhibit or repress their own biosynthesis metabolic engineering approaches can be used to achieve overproduction of the secondary metabolites. One general strategy is to increase the step after formation of the inhibitory metabolite, and as discussed before, increased secretion is a good option. Alternatively, genes encoding feedback-insensitive enzymes can be introduced in the producing organisms (Sanchez and Demain, 2002).

Understanding of the compartmentalization of a biosynthetic-pathway can also provide novel strategies for metabolic engineering of the secondary-metabolite producing organisms, and guide an expression of the pathways in a heterologous host. As exemplified by the finding that co-localization of the penicillin biosynthetic enzymes in peroxisomes improved penicillin production in *Aspergillus nidulans* (Herr and Fischer, 2014). The three main enzymes required for the synthesis of penicillin are located in different subcellular compartments. Whereas the nonribosomal peptide synthase AcvA and the isopenicillin N (IPN) synthase IpnA localize to cytoplasm, the last enzyme that converts IPN to penicillin G, namely AatA, resides in peroxisomes. Targeting of AcvA to peroxisomes led to a 3.2-fold increase in penicillin yield. Moreover, the number of peroxisomes was doubled, resulting in 2.3 times higher production of penicillin.

Improvement of cell factories by increasing their resistance to the produced metabolites cannot always be achieved by overexpression the self-resistance genes or by modification of feedback regulation or compartmentalization, as they are often unknown. In these cases, other approaches have to be used to construct the strains that can tolerate high concentrations of the toxic compounds. Random mutagenesis or genome shuffling (Zhang et al., 2002a) are some of the possible approaches. The latter was successfully used to improve the self-resistance to antibiotic pristinamycin in *Streptomyces pristinaespiralis*. Strain with improved resistance also exhibited 1.9-fold increase in the production of this antibiotic compared to the wild type strain (Xu et al., 2008). Moreover, target engineering (i.e. generating mutations that confer drug resistance) (Ochi, 2007) improved production of several antibiotics in *Streptomyces spp*. (Hesketh, 1997; Shima et al., 1996; Tanaka et al., 2009; G. Wang et al., 2008)

If bioactive secondary metabolite is to be produced in a heterologous host, a resistance mechanism will be necessary to overcome the product toxicity where the target of the produced compound is present in the host organism. Genes encoding for the self-resistance mechanisms in the natural producers are good candidates for

co-expression in the host microorganism. However, it is not likely that all types of self-resistance mechanisms are equally easy to transfer to a heterologous host, e.g. methylation of rRNA might not be compatible with other organisms. Understanding the self-resistance mechanism in a natural producer can also guide the engineering of the heterologous host. Improvement of the secondary-metabolite resistance has been shown to be a good metabolic engineering strategy towards increased production of the compound in a heterologous host; *de novo* biosynthesis of vanillin in *Schizosaccharomyces pombe* was improved by introduction of UDP-glycosyltransferase from the plant *Arabidopsis thaliana*, an enzyme that converts vanillin, which is toxic to yeast, to nontoxic vanillin β-D-glucoside (Hansen et al., 2009). In conclusion, a growing body of evidence suggests that the use of both, self-resistance mechanisms as well as other strategies for improved resistance can improve yields of secondary metabolites, and thereby have a potential to significantly improve biotechnological processes for their production.

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PART TWO (Experimental Work)

CHAPTER 4

Attempt to construct a *Saccharomyces cerevisiae* cell factory for production of compactin

The goal of this subproject was to express the *P. citrinum* compactin biosynthetic pathway in a heterologous host, i.e. *Saccharomyces cerevisiae*. This ambition was not accomplished, nevertheless, in this chapter I will briefly explain the reasoning behind the choice of organism, and provide a summary of the work aimed at constructing the yeast-based compactin cell factory, as it places the further experimental chapters (Chapters 5 and 6) into a perspective.

4.1 Introduction

As presented in Chapter 2, the biotechnological production of natural and semi-natural statins is based on submerged fermentation of filamentous fungi, such as *Aspergillus spp*. and *Penicillium spp*.. The filamentous growth of these organisms lead to formation of dense pellets that increase viscosity and reduce oxygen solubility, which make their cultivation in standard liquid fermentation systems difficult and expensive. Moreover, statins are synthesized when the fungal growth is limited, and the nutrients are exhausted (Hajjaj et al., 2001), which increases the difficulty of the fermentation process control. The filamentous fungi used for production are also capable of synthesizing numerous other secondary metabolites; a feature that can reduce yields and complicate the product purification steps. One example of an undesirable co-metabolite during lovastatin fermentation in *A. terreus* is benzophenone sulochrin (Couch and Gaucher, 2004). In order to be commercially

successful, statins produced by fermentation must be cost-competitive; therefore there is a constant need for improvement of the production processes.

Optimization of the biotechnological production of natural and semi-natural statins has mostly been based on two approaches; optimization of the bioprocessing conditions and improvement of the production organism (Barrios-González and Miranda, 2010; Mulder et al., 2015; S. K. Singh and Pandey, 2013). However, strategies to improve secondary metabolite production can also include the transfer of the biosynthetic gene cluster to a heterologous host. Heterologous production of statins in a fast-growing host, such as *S. cerevisiae*, could bring new opportunities for optimization of this process, thus making it cost-competitive. Moreover, heterologous production of statins would provide a platform for future combinatorial biochemistry experiments aimed at the synthesis of novel statins with improved pharmacokinetic and pharmacodynamics properties.

The yeast *S. cerevisiae*, a unicellular eukaryotic organism from the kingdom of fungi, is one of the most widely used cell factories (Borodina and Nielsen, 2014; Nielsen and Jewett, 2008). *S. cerevisiae* is suitable for large-scale production of various products because of its high specific growth rates, and high biomass levels on simple and cheap media, and on complex (rich) and defined (minimal) media. Besides the ease of cultivation, *S. cerevisiae* is well characterized both physiologically and genetically, enabling optimization of existing biotechnological production processes using this organism as a heterologous production host. The broad range of available tools for genetic manipulation of *S. cerevisiae* allows for easier genetic engineering than it is possible in e.g. filamentous fungi (Da Silva and Srikrishnan, 2012). Advancements in synthetic biology and metabolic engineering tools have enabled and enhanced our ability to engineer *S. cerevisiae* to produce a wide variety of value-added products, including polyketides, e.g. 6-methylsalicylic acid (6-MSA) from *Penicillium patulum* (Kealey et al., 1998; Wattanachaisaereekul et al., 2008, 2007), (*R*)-monocillin II from *Pochonia chlamydosporia* (Zhou et al., 2010b), and rubrofusarin from *Fusarium graminearum* (Rugbjerg et al., 2013). Despite the

obvious advantages of *S. cerevisiae* as a host for industrial applications, and its capacity to synthesize a diverse range of products, several requirements need to be considered when *S. cerevisiae* is intended for production of polyketides (e.g. statins). Firstly, heterologous production of polyketides requires the functional expression and correct posttranslational modifications of the large and complex enzymes, i.e. PKSs. In order to be functional, PKSs require posttranslational modification by 4'-phosphopantetheinyl transferase (PPTase), which covalently attaches the 4'-phosphopantetheine (P-pant) moiety of CoA to a conserved serine residue in the P-pant-dependent carrier protein of PKS. Therefore, co-expression of PPTase with PKSs is essential for the production of polyketides in *S. cerevisiae* (Wattanachaisaereekul et al., 2007). Secondly, building blocks for the polyketide biosynthesis must be available in sufficient quantities to sustain high production levels. Thirdly, *S. cerevisiae* must possess a resistance mechanism against any toxic effects the produced compound(s) may have. The subject of resistance was already introduced in Chapter 3, and will be further discussed in Chapters 5 and 6. Here I provide a summary of the work aiming at the heterologous expression of the compactin biosynthetic pathway (Figure 1) in *S. cerevisiae*.

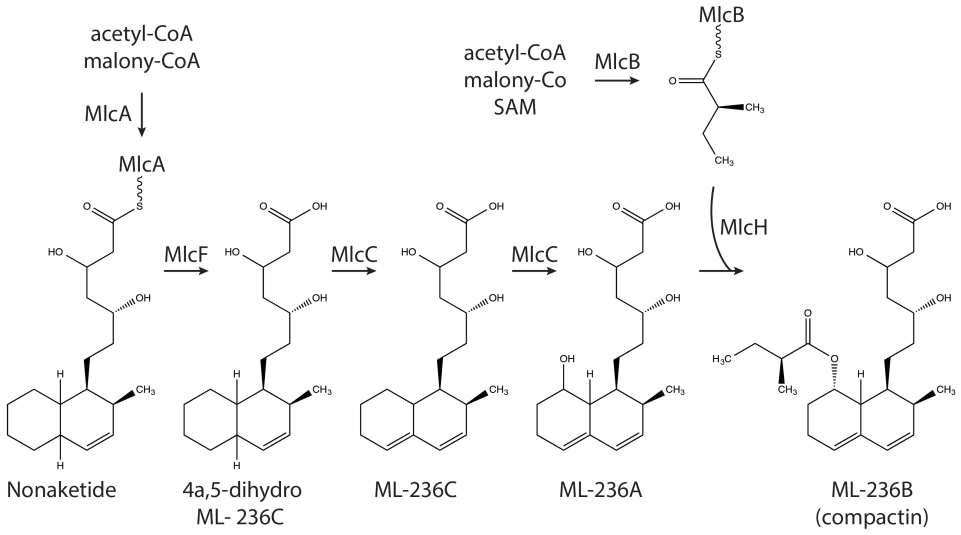

Figure 1: Compactin biosynthetic pathway as proposed by Abe et al (Abe et al., 2002b), and according to the new information regarding the lovastatin pathway (Xu et al., 2013). MlcA – nonaketide synthase, MlcB – diketide synthase, MlcC – P450 monooxygenase, MlcF – oxidoreductase, MlcG – enoyl reductase, MlcH – Transesterase, SAM – S-adenosylmethionine.

4.2 Results and discussion

The attempt to construct a *S. cerevisiae* based cell factory for compactin production involved the expression of the compactin biosynthetic genes from *P. citrinum* in *S. cerevisiae* using a chromosomal gene integration approach. Stable integration is more suitable for the introduction of multiple genes, and thus expression of metabolic pathways, compared to the autonomously replicating plasmid vectors, because it offers precise gene copy number control and long-term stability (Da Silva and Srikrishnan, 2012). The use of vectors, such as 2μ or CEN/ARS, can result in loss of the required genes, as two or more copies of these vectors can be difficult to stably maintain in a single cell (Futcher and Cox, 1983; Mead et al., 1986). Moreover, expression of multiple genes from integrative cassettes is more homogenous within the yeast population than expression from multiple plasmid vectors (Jensen et al., 2014). For genomic integration of the compactin biosynthetic genes, the yeast expression platform developed by Mikkelsen et al. (University of Copenhagen, Denmark) was applied (Mikkelsen et al., 2012). Codon-optimized *de novo* synthesized compactin biosynthetic genes (*mlcA, mlcC, mlcF* and *mlcG*; Table 1) required for the synthesis of the nonaketide intermediate ML-236A (Figure 1) were expressed in *S. cerevisiae* CEN.PK113-11C as single copy genes from defined loci in the yeast genome (Table 1). Strong constitutive promoters *TEF1* or *PGK1* were used for expression of all genes, except for the PKS-encoding gene *mlcA*, which was expressed from the galactose-inducible promoter *GAL10* (Table 1). In order to avoid self-intoxication, two putative self-resistance genes *mlcD* and *mlcE* were co-expressed, resulting in a strain with six genes from the compactin biosynthetic gene cluster. To ensure efficient post-transcriptional activation of the MlcA PKS, two genes encoding for PPTase with broad substrate specificity were co-expressed individually in the pathway-harboring yeast strain; either *npgA* from *Aspergillus nidulans* (Keszenman-Pereyra et al., 2003), or *sfp* from *Bacillus subtilis* (Mootz et al., 2001), resulting in strains AR22 and AR23,

respectively. The constructed strains (AR22 and AR23) were analyzed for the production of expected intermediates (4a,5-dihydro ML236-C, ML236-C or ML-236A) in the compactin biosynthetic pathway (Figure 1) by LC-MS, however, we were not able to detect any new compounds in the analyzed strains (AR22 and AR23) (data not shown). The method used for extraction and analysis of the expected intermediates have been previously verified (Kildegaard, 2011), ruling out the possibility that the negative results were due to inadequate procedures to extract and detect the metabolites.

Table 1

List of genes integrated into the *S. cerevisiae* CEN.PK genome using the yeast expression platform developed by Mikkelsen et al (Mikkelsen et al., 2012).

Gene name	(Putative) function	Promoter	Integration site[a]
Genes from the *P. citrinum* compactin biosynthetic gene cluster			
mlcA	Nonaketide synthase (Synthesis of the polyketide backbone)	*GAL10*	X-4
mlcC	P450 monooxygenase (hydroxylation and dehydration of the backbone)	*TEF1*	XII-1
mlcF	Oxidoreductase (Release of the intermediate from MlcA)	*PGK1*	XII-1
mlcG	Enoyl reductase (Reduction of the nonaketide backbone)	*TEF1*	XII-4
mlcD	HMG-CoA reductase-like (Resistance)	*PGK1*	X-3
mlcE	Efflux pump (Resistance)	*TEF1*	X-3
Genes for posttranslational modification of MlcA			
npgA	PPTase (activation of MlcA)	*TEF1*	X-2
sfp	PPTase (activation of MlcA)	*TEF1*	X-2

[a] Roman numerals indicate the number of a chromosome, and whole numbers indicate the locus on a chromosome, in which the gene was integrated.

To test whether the gene-integration and induction regime allowed for the expression of the key-enzyme in the pathway, PKS MlcA, I tagged *mlcA* with RFP at its carboxylic terminus. Fluorescent microscopy of the resulting strain (AR30) revealed that MlcA indeed was expressed and that the fluorescent signal was

accumulated as bright foci in the cells (Figure 2). This result indicates that MlcA is either localized in a certain subcellular compartment or is accumulated as protein aggregates (inclusion bodies) in the cytosol, which may arise from improperly folded, and thus insoluble proteins (Fink, 1998; Y. Wang et al., 2009). Both situations could explain the inability of the recombinant strains (AR22 and AR23) to produce the intermediates of the compactin biosynthetic pathway. If the PKS MlcA is localized in a compartment separately from the rest of the required enzymes (i.e. PPTase), it is unable to synthesize the nonaketide backbone. More specifically, MlcA would be inactive in the absence of PPTase due to the lack of posttranslational modification. A similar phenotype could also be result from the incompatibility of the chosen PPTases with MlcA, however both NpgA and Sfp had previously been proven to be a suitable candidates for heterologous co-expression with various PKSs (Quadri et al., 1998; Wattanachaisaereekul et al., 2007), including the lovastatin nonaketide synthase LovB (Ma et al., 2009a). Moreover, insufficient availability of biosynthetic substrates (i.e. acetyl-CoA, malonyl-CoA and S-adenosylmethionine) in specific cellular compartments may be insufficient. The precursor supply for polyketide production in *S. cerevisiae* has been shown to be important when establishing a *S. cerevisiae* cell factory for 6-MSA production (Wattanachaisaereekul et al., 2008). Improper folding of MlcA could also provide an explanation for the failure to produce compactin intermediates, as misfolded proteins may possess insufficient catalytic activity (Fink, 1998). The results shown in Figure 2 are very similar to those published by Siewers et al., where heterologous expression of a non-ribosomal peptide synthase (NRPS) in *S. cerevisiae* resulted in formation of protein aggregates that appeared as bright foci when the NRPS was fused with YFP at its C-terminus (Siewers et al., 2009).

One of the factors affecting the protein folding is the cultivation temperature, and its reduction can enable the cells to fold the proteins correctly (Gidijala et al., 2008; Li et al., 2001). In our case, lowering the cultivation temperature from 30°C to 25°C or 20°C did not result in production of compactin intermediates in the AR22

and AR23 strains, nor did it affect the observed intracellular distribution of RFP-tagged MlcA (data not shown). These results did not resolve the question, why the constructed strains were not producing the expected compactin intermediates. Further investigation of the expression of the compactin biosynthetic genes, the intracellular localization of the heterologous proteins and their posttranslational modification (i.e. phosphopantetheinylation of MlcA) is required, and an alternative expression system could be considered.

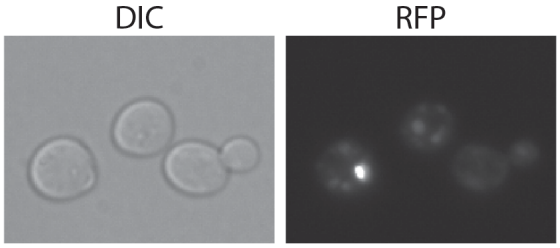

Figure 2: Fluorescence microscopy of *S. cerevisiae* expressing mlcA-RFP (strain AR30; *X4::(pGAL10-mlcA-RFP)*). Prior to microscopy the strain was cultured in liquid YPGal medium at 30°C with 150 rpm agitation overnight. DIC – differential interference contrast.

4.3 Conclusions

Heterologous production of statins in a fast-growing host, such as *S. cerevisiae*, has been an ambition of several research groups around the world. The intention to construct a *S. cerevisiae* cell factory for compactin production was not realized, as the yeast strains harboring the compactin biosynthetic genes did not produce any of the expected intermediates in the compactin pathway. At the time when I obtained the results described in this chapter, another research group, namely Xu et al. (University of California, Los Angeles, USA), succeeded in producing the first stable lovastatin intermediate dihydromonacolin L acid in *S. cerevisiae* (Xu et al., 2013). They used plasmid vectors to express LovB (nonaketide synthase), LovG (oxidoreductase) and LovC (enoyl reductase) in *S. cerevisiae* strain NpgA BJ5464-NpgA (Ma et al., 2009b), which is a vacuolar protease-deficient yeast strain with the *A. nidulans npgA* integrated chromosomally (Mootz, 2002). Besides

the different expression system, the deletion of two vacuolar proteases (*PEP4* – vacuolar aspartyl proteinase A, and *PRB1* – vacuolar proteinase B) in the expression strain that was used in their study could have a major impact on the successful expression of the lovastatin biosynthetic enzymes as proteases, especially luminal vacuolar proteases, can significantly affect protein production in yeast, and can generate artifacts concerning the structure, activity as well as localization of proteins in the cells (Jones, 1991). The results from Xu et. al showed that a future yeast-based production of statins is possible. This achievement will open numerous opportunities for engineering *S. cerevisiae* strains aimed at improved production of statins. Solutions for tackling the problem with *S. cerevisiae* statin sensitivity could have an enormous impact on future production of statins in yeast, which is why I decided to switch focus from the cell-factory construction to establishing statin resistance and export in *S. cerevisiae* as described in the following two chapters.

4.4 Materials and Methods

4.4.1 Plasmid and yeast strain construction

Yeast codon-optimized versions of the biosynthetic genes from the *P. citrinum* compactin gene cluster (Table 1), *de novo* synthetized by Genscript, were PCR amplified from the relevant plasmids (Table 2) using primer pairs as listed in Table 4. PPTase-encoding genes *npgA* and *sfp* were amplified from plasmids pnpgATNT2 and p424sfp using primer pairs npgA-F/npgA-R and sfp-F/sfp-R, respectively. The PGK1 and TEF1 promoter sequences were amplified from plasmid pSP-G2, and *GAL10* was amplified from pESC-Ura using primer pair GalP-F2 and GalP-R. Expression of MlcA was analyzed by tagging it C-terminally with monomeric red fluorescent protein (RFP). For this, the plasmid pX4-GalP-mlcA was constructed, by amplifying *mlcA* coding sequence lacking the stop codon, using the primer pair

mlcA-F/mlcA-CO-RFP-R, and yeast codon-optimized *RFP* was amplified from plasmid pWJ1350 using primer pair RFP_F+/RFPR+.

The amplified fragments were cloned into the targeting vectors (Table 2) (Mikkelsen et al., 2012) via the USER (uracil-specific excision reagent) cloning technique (Nour-Eldin et al., 2006) resulting in plasmids carrying the gene expression cassettes. All fragments were amplified by PCR using a 2-deoxyuridine compatible PfuX7 polymerase (Nørholm, 2010). *Escherichia coli* DH5α (Woodcock et al., 1989) was used as host for USER cloning experiments and for propagation of the constructed plasmid. The constructed plasmids were digested with the NotI enzyme (New England Biolabs), and the obtained linear fragments were introduced into yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol transformation (Gietz and Schiestl, 2007). The linear gene targeting cassettes were integrated into the different loci of the reference yeast strain, *S. cerevisiae* CEN.PK 113-11C as described by Mikkelsen et al (Mikkelsen et al., 2012). Integration sites into which the individual genes were integrated are listed in Table 1. Correct integration of the substrates was verified by diagnostic colony PCR with one primer annealing outside of the integration site in the yeast genome (e.g. X-3-up-out-sq for confirmation of the correct integration of the expression cassette in the integration site 3 on chromosome X), and one substrate specific primer (C1_TADH1_F) annealing within the integrated targeting cassette. Strains used in this study are listed in Table 3.

Table 2

List of plasmids used in this study.

Plasmid name	Description	Reference or source
pEN669	Template for amplifying *mlcE* (*S. cerevisiae* codon optimized)	from Evolva Holding SA
pEN668	Template for amplifying *mlcD* (*S. cerevisiae* codon optimized)	from Evolva Holding SA
pEN155	Template for amplifying *mlcG* (*S. cerevisiae* codon optimized)	from Evolva Holding SA
pEN156	Template for amplifying *mlcF* (*S. cerevisiae* codon optimized)	from Evolva Holding SA
pEN157	Template for amplifying *mlcA* (*S. cerevisiae* codon optimized)	from Evolva Holding SA
pEN158	Template for amplifying *mlcC* (*S. cerevisiae* codon optimized)	from Evolva Holding SA
pnpgATNT2	Template for amplifying *npgA*	Louise Mølgaard, Novo Nordisk A/S, Denmark
p424sfp	Template for amplifying *sfp*	(Wattanachaisaereekul et al., 2007)
pWJ1350	Template for amplifying *RFP*	(Lisby et al., 2003)
pSP-G2	Template for amplifying *TEF1* and PGK1	(Partow et al., 2010)
pESC-Ura	Template for amplifying *GAL10*	Tomas Strucko, Technical University of Denmark
pX2	USER cloning vector equipped with the *CYC1* and *ADH1* terminators designed to target site 2 on chromosome X.	(Mikkelsen et al., 2012)
pX3	USER cloning vector equipped with the *CYC1* and *ADH1* terminators designed to target site 3 on chromosome X.	(Mikkelsen et al., 2012)
pX4	USER cloning vector equipped with the *CYC1* and *ADH1* terminators designed to target site 4 on chromosome X.	(Mikkelsen et al., 2012)
pXII1	USER cloning vector equipped with the *CYC1* and *ADH1* terminators designed to target site 1 on chromosome XII.	(Mikkelsen et al., 2012)
pXII4	USER cloning vector equipped with the *CYC1* and *ADH1* terminators designed to target site 4 on chromosome XII.	(Mikkelsen et al., 2012)
pX2-TEF1-sfp	Plasmid carrying a gene-targeting cassette for expressing *sfp* in yeast.	This study
pX2-TEF1-npgA	Plasmid carrying a gene-targeting cassette for expressing *npgA* in yeast.	This study
pX3-mlcD-PGK1-TEF1-mlcE	Plasmid carrying a gene-targeting cassette for expressing *mlcD* and *mlcE* in yeast.	This study
pX4-GalP-mlcA	Plasmid carrying a gene-targeting cassette for expressing *mlcA* in yeast.	This study
pX4-GalP-mlcA-RFP	Plasmid carrying a gene-targeting cassette for expressing *mlcA-RFP* in yeast.	This study
pXII1-mlcF-PGK1-TEF1-mlcC	Plasmid carrying a gene-targeting cassette for expressing *mlcC* and *mlcF* in yeast.	This study
pXII4-TEF1-mlcG	Plasmid carrying a gene-targeting cassette for expressing *mlcG* in yeast.	This study

Table 3

List of strains used in this study.

Strain name	Genotype	Reference or source
Escherichia coli		
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	(Woodcock et al., 1989)
Saccharomyces cerevisiae		
CEN.PK113-11C *(Wild type strain)*	*MATα MAL2-8C SUC2 his3Δ ura3-52*	Dr. Petter Kötter, Institut für Mikrobiologie, der Johan Wolfgang Goethe-Universität, Frankfurt am Main, Germany
AR22	*MATα MAL2-8C SUC2 his3Δ ura3-52 X2::(pTEF1-npgA) X3::(pPGK1-mlcD, pTEF1-mlcE) X4::(pGAL10-mlcA) XII1::(pPGK1-mlcF, pTEF1-mlcC) XII-4::(pPGK1-mlcG)*	This study
AR23	*MATα MAL2-8C SUC2 his3Δ ura3-52 X2::(pTEF1-sfp) X3::(pPGK1-mlcD, pTEF1-mlcE) X4::(pGAL10-mlcA) XII1::(pPGK1-mlcF, pTEF1-mlcC) XII-4::(pPGK1-mlcG)*	This study
AR31	*MATα MAL2-8C SUC2 his3Δ ura3-52 X2::(pTEF1-npgA) X4::(pGAL10-mlcA-RFP)*	This study

Table 4

List of oligonucleotides used in this study. U = 2-deoxyuridine.

Primer name	Primer sequence (5`- 3`)	Use
mlcD-F	ATCAACGGGUAAAAATGGTGGCTTCCT	Amplification of *mlcD* from plasmid pEN668
mlcD-R	CGTGCGAUTCAACGTCTGGCAC	
mlcA-F	ATCCGTAATACUAAAAATGGATCAAGCTAATTATC	Amplification of *mlcA* from plasmid pEN157
mlcA-R	CACGCGAUCTAAGCTAACTTAAGTC	
mlcE-F	AGCGATACGUAAAAATGAGTGAACCATTACC	Amplification of *mlcE* from plasmid pEN669
mlcE-R	CACGCGAUTTATGCATCAGTCTCAG	
mlcC-F	AGCGATACGUAAAAATGTTGGGTCAAGTTTTATTGACTG TAG	Amplification of *mlcC* from plasmid pEN158
mlcC-R	CACGCGAUTTAGCATCTGTCATGTGGTAGTGGA	
mlcF-F	ATCAACGGGUAAAAATGTCTCCAGCTAGAATTAC	Amplification of *mlcF* from plasmid pEN156
mlcF-R	CGTGCGAUCTACACGAAAGATCCACT	
mlcG-F	AGCGATACGUAAAAATGGGTGTTGCTATGACTGA	Amplification of *mlcG* from plasmid pEN155
mlcG-R	CACGCGAUTTAGACAGAGAACCTTACAACG	
npgA-F	ATCAACGGGUAAAAATGGTGCAAGACACATC	Amplification of *npgA* from plasmid pnpgATNT2
npgA-R	CGTGCGAUTTAGGATAGGCAATTACACA	
Sfp-F	ATCAACGGGUAAAAATGAAGATTTACGGAAT	Amplification of *sfp* from plasmid p424sfp
Sfp-R	CGTGCGAUTTATAAAAGCTCTTCG	
TEF1-d	ACGTATCGCUGTGAGTCGTATTACGGATCCTTG	Amplification of promoter sequences from plasmid pSP-G2
PGK1-s	CGTGCGAUGCCGCTTGTTTTATATTTGTTG	
TEF1-s	CACGCGAUGTGAGTCGTATTACGGATCCTTG	
PGK1-d	ACCCGTTGAUGCCGCTTGTTTTATATTTGTTG	
GalP-F2	AGCCCTTTAGUGAGGGTTGAATTCGAAT	Amplification of promoter sequence from plasmid pESC-Ura
Gal-R	AGTATTACGGAUCCGGGGTTT	
RFP_F+	ATGGCCTCCUCCGAGGACGTCATCAAGGAG	Amplification of *RFP* sequence from plasmid pWJ1350
RFPR+	CACGCGAUCTAGGCGCCGGTGGAGTGGCGG	
mlcA-CO-RFP-R	AGGAGGCCAUAGCTAACTTAAGTGCAGGGTTCATA	pX-4 GalP mlcA (CO Evolva)
X-2-up-out-sq	TGCGACAGAAGAAAGGGAAG	Strain confirmation via colony PCR
X-3-up-out-sq	TGACGAATCGTTAGGCACAG	
X-4-up-out-sq	CTCACAAAGGGACGAATCCT	
XII-1-up-out-sq	CTGGCAAGAGAACCACCAAT	
XII-4-up-out-sq	GAACTGACGTCGAAGGCTCT	
C1_TADH1_F	CTTGAGTAACTCTTTCCTGTA	

4.4.2 Media

The *E. coli* transformants were selected on lysogeny broth (LB) medium containing 100 µg/mL of ampicillin. Yeast strains were cultivated in standard liquid or solid yeast peptone dextrose medium (YPD; 10 g/L of yeast extract, 20 g/L of peptone and 20 g/L of glucose), synthetic complete medium (SC), or YPD medium in which glucose was replaced by 2% galactose as a carbon source, thus named YPGal. SC medium was prepared according to Sherman et al. (Sherman et al., 1986), with the minor modification that the L-leucine concentration was doubled to 60 mg/L. Yeast transformants were selected on SC medium lacking uracil. Removal of the *URA3* marker, via direct repeat recombination, was achieved by growing the strain on SC medium containing 5-fluororotic acid (5-FOA; 740 mg/L, Melford) and uracil (30 mg/L).

4.4.3 Fluorescent Microscopy

For fluorescent microscopy the *mlcA-RFP* expressing strain (AR31; X4::*(pGAL10-mlcA-RFP)*) was cultured in liquid YPGal medium at 30°C, 25°C or 20°C with 150 rpm agitation overnight and analyzed by fluorescence and visible light microscopy using a Nikon Eclipse E1000 microscope equipped with an oil-immersed objective at 100 x magnification. The images were captured with QImaging Retiga Exi digital camera using Image Pro Plus 5.1 software. The brightness of images to be compared was adjusted pairwise using Adobe Photoshop CS6.

4.4.4 Metabolite extraction and detection

Yeast strains were grown in 100 mL YPGal medium in 500 mL shake flasks at 30°C, 25°C or 20°C and 150 rpm for 2 days. After incubation, 50 mL of the culture

broth were transferred to 50 mL Falcon tubes, and the cells were separated form the broth by centrifugation (10 min, 4500 rpm). Metabolites were extracted both from the supernatant and the cell pellet. Supernatant (10 mL) was first acidified to pH 3 with 2 M HCl and then extracted with 10 mL of ethyl acetate containing 0.5 % formic acid at room temperature for 4 hours (lightly shaking on an orbital shaker). After extraction, 5 mL of the ethyl acetate phase was transferred to a new Falcon tube, the extract was dried under a stream of nitrogen, re-dissolved in 0.5 mL methanol and ultrasonicated for 10 min. Finally, the dissolved extract was filtered through a 0.45 µm Minisart RC4 filter (Sartorius, Germany) and subjected to LC-MS analysis. For extraction of metabolites from the cells, the cell pellet was first re-dissolved in 1 mL of MilliQ water and then disrupted using glass beads and a Savant FastPrep FP120 cell disrupter system. 1 mL of ethyl acetate containing 0.5 % formic acid were added to the disrupted cells, and the extraction took place at room temperature for 4 hours (lightly shaking on an orbital shaker). The cells and glass beads were separated from the extraction solvent by centrifugation (10 min, 4500 rpm), and 0.5 mL of the ethyl acetate phase were subjected to the drying, re-dissolving, sonication and filtering procedure as described above. LC-MS was carried out on Ultima 3000 UHPLC (Dionex, Sunnyvale, CA) coupled to a MaXis G3 (Bruker Daltronics, Bremen, Germany) high-resolution mass spectrometer. Separation was achieved on a Kinetic C18 column (Phenomenex, Torrance, CA). The column was eluted using A: Water containing 20 mM formic acid and B: MeCN containing 20 mM formic acid using a linear gradient from 10 % B to 100 % B in 10 min. All samples were calibrated by infusing of NaHCOO prior to all injections using Bruker HPC calibration algorithm achieving a mass accuracy < 1 ppm. The MS was operated in positive ESI mode.

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CHAPTER 5

MlcD – a metabolic backup system to the standard 3-hydroxy-3-methylglutaryl coenzyme A reductase in statin producing fungi

5.1 Introduction

In their natural habitats filamentous fungi face tough competition for the limited nutrition resources that are available. Prokaryotic competitors are typically combatted by fungi through excretion of potent antibiotics, which target unique prokaryotic molecular features to give the producing fungi a competitive advantage. The large differences in essential enzymatic systems that exist between prokaryotes and eukaryotes offers plenty of opportunities for the development of bioactive secondary metabolites that can inhibit prokaryotes without negatively affecting the eukaryotic producer. Penicillin, a secondary metabolite synthesized by *Penicillium spp.* is the cardinal example of this. In addition to the prokaryotic competitors, fungi often also face eukaryotic adversaries in the fight for survival, either in the form of other fungi adapted to utilize the same nutritional sources, or invertebrates that eat fungi. In these cases, the effective use of bioactive compounds in the confrontation is a bit more complex, and challenging in an evolutionary perspective, as both partners are eukaryotes and hence share many fundamental cellular processes. This situation significantly reduces the number of unique targets that exist in either of the adversaries, reducing the likelihood that biosynthetic pathways for the formation of efficient bioactive compounds will evolve (it will take longer time).

One way of escaping this curse of shared ancestry is for the organism to produce bioactive compounds that affect shared molecular targets, but which affect the competitor(s) at least marginally more than they affect the producing organism,

or which are first activated upon release from the producer. This will allow the producer to subsequently gain an additional competitive advantage by evolving/gaining a mechanism to avoid self-intoxication, which in turn will give room for the evolution of higher production levels.

Currently only a limited number of examples of this evolutionary solution has been documented in the fungal world[4]. In the published cases, the gene encoding for the self-resistance mechanism have been found to cluster together with the genes encoding the biosynthetic machinery for formation of the bioactive secondary metabolites (Brakhage, 2013; Smith et al., 1990). One example of this is *Penicillium brevicompactums* production of mycophenolic acid (MPA), an inhibitor of eukaryotic inosine-5`-monophosphate dehydrogenase (IMPD). *P. brevicompactum* contains two genes encoding for IMPD, PbIMPDH-A and PbIMPDH-B (also called *mpaF*), the latter being located within the MPA biosynthetic gene cluster (Regueira et al., 2011). Interestingly, MpaF is remarkably resistant to MPA, therefore it has been suggested that it is the key component of MPA self-resistance mechanism in *P. brevicompactum* (Hansen et al., 2011; Sun et al., 2011). Statins are another example of a eukaryotic toxin produced by fungi. The exact function of these compounds in nature has not been proven, but it is well known that statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), an enzyme that catalyzes the rate-limiting step in the mevalonate pathway-derived biosynthesis of nonsterol isoprenoids and sterols, such as cholesterol and ergosterol (Maury et al., 2005). The first statin to be isolated was compactin (also called compactin or ML-236B) from cultures of *Penicillium citrinum* (Endo et al., 1976b). Since the target of compactin, HMGCR, is also present in the producing fungus, the latter must possess a mechanism of self-protection to overcome the inhibition of the mevalonate pathway. Characterization of the compactin biosynthetic gene cluster revealed the presence of a putative self-resistance gene encoding for an HMGCR-like protein, namely *mlcD* (Abe et al., 2002b). The involvement of this gene in the

[4] A large number of secondary metabolites produced by fungi are toxic to vertebrates (mycotoxin group), however in the majority of cases this situation should likely be considered as collateral damage (as unfortunate side effect) as non-pathogenic fungi would not gain (in an evolutionary perspective) from killing vertebrates.

self-resistance mechanism was suggested when a *P. citrinum* compactin-sensitive mutant was isolated after cosmid-mediated recombination aimed at improved production of compactin (Abe et al., 2002a). Characterization of this mutant showed that a region containing the *mlcD* locus was not present in the sensitive strain. Homologs of *mlcD*, *lvrA* and *mokG* are found in the lovastatin gene cluster from *Aspergillus terreus* (Alberts et al., 1980) and *Monascus pilosus* (Chen et al., 2008), respectively. MlcD (1173 aa) and LvrA (1068 aa) display 40.1% identity and 55.1% similarity[5] at amino acid levels. Protein identity (73%) and similarity (82%) are even higher between LvrA and MokG (1052 aa). All three enzymes possess the same domain organization (Figure 1). Hutchinson et al. reported that introduction of *lvrA* into the lovastatin sensitive *Aspergillus nidulans* resulted in increased lovastatin resistance, however, this statement was not supported by experimental data in the publication (unpublished results) (Hutchinson et al., 2000). Collectively, the available information leaves the molecular basis of the compactin self-resistance mechanism unresolved.

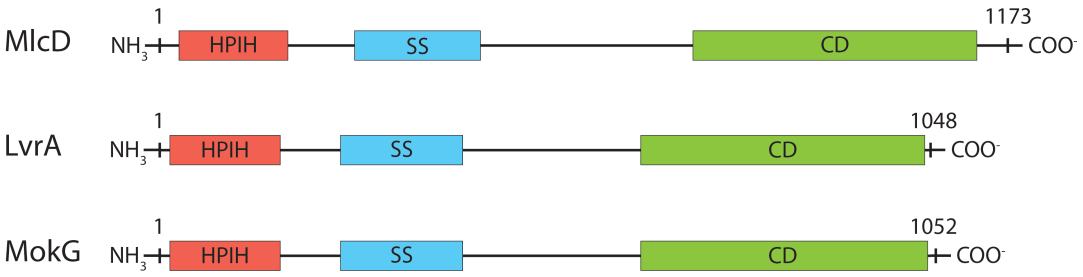

Figure 1: Domain organization of HMGCRs; MlcD from *P. citrinum*, LvrA from *A. terreus*, and MokG from *M. pilosus* HPIH – N-terminal domain with HPIH motif (PF13323.1; function unknown); SS – sterol sensing domain (PF12349.3); CD – catalytic domain (PF00368.13; "HMG-CoA reductase"). Domains were identified using Pfam protein family database version 27.0 (Finn et al., 2014).

The discovery that statins can be used to modify and control cholesterol levels in vertebrates, by inhibiting *de novo* synthesis, has had an enormous impact on human health and treatment of cardiovascular diseases (Corsini et al., 1995).

[5]Identity and similarity were calculated using EMBOSS Needle pairwise global sequence alignment tool (Rice et al., 2000).

Industrial scale production of natural statins is based on fermentation of *Aspergillus* and *Penicillium spp.,* and many different strategies to increase the fermentation titers have already been applied (Barrios-González and Miranda, 2010). With respect to production-strain engineering aimed at increased production of secondary metabolites in the natural producer, understanding of the mechanisms of self-resistance has in several instances been shown to be important (Lomovskaya et al., 1996; Malla et al., 2010; Ullán et al., 2002; Zhang et al., 2002b). This is also the case for statin formation where one of the attempts to improve lovastatin production in *A. terreus* lead to the isolation of a mutant which showed a 3-fold increase in the production of lovastatin compared to the parental strain caused by increased resistance to lovastatin (Jia et al., 2011). This suggests that a better understanding of the mechanisms underlying the statin self-resistance likely can provide novel genetic engineering strategies to reach higher statin titer. In addition, it also opens up for the possibility to produce statins in a heterologous host, such as *Saccharomyces cerevisiae*, that is sensitive.

In this work we aimed to elucidate the function of *mlcD* gene from the *P. citrinum* compactin biosynthetic gene cluster. We used the statin-sensitive *Saccharomyces cerevisiae* as a model host to examine if MlcD can confer the statin-resistance, and in hope of gaining insight into the molecular basis behind the resistance.

5.2 Results and Discussion

5.2.1 *mlcD* confers the resistance to statins in yeast

S. cerevisiae has previously been shown to be sensitive to statins present in the growth medium (Formenti and Kielland-Brandt, 2011; Rine et al., 1983), leading to the use of *S. cerevisiae* as a model organism for mapping the mode of action for

statins at the cellular level (Callegari et al., 2010). Statins inhibit the activity of yeast HMGCRs, ultimately resulting in ergosterol starvation and lack of protein prenylation, causing reduced cell viability and mitochondrial dysfunction (Callegari et al., 2010). To confirm the hypothesis that the *mlcD* gene from the *P. citrinum* compactin biosynthetic gene cluster can confer the resistance to statins, we expressed *mlcD* as a single copy gene under the constitutive promoter TEF1 from a defined genomic locus in *S. cerevisiae* (ARX4 strain). The strain's sensitivity to statins was assayed via a growth experiment on solid YPD medium with increasing concentrations of lovastatin, the compactin structural analog. The *mlcD*-expressing strain displayed an increased resistance to lovastatin present in the medium, compared to the reference wild-type strain (Figure 2). This result shows that MlcD can confer the resistance to the compactin structural analog, lovastatin, when expressed in a heterologous host *S. cerevisiae*. This also supports the hypothesis by Abe et al. that *mlcD* encodes for a statin-resistance mechanism in its native host.

5.2.2 MlcD is an HMG-CoA reductase (HMGCR)

MlcD and LvrA have previously been suggested to be HMGCRs capable of converting HMG-CoA into mevalonate, based on sequence similarity and domain architecture, however this suggestion has not been confirmed experimentally so far. To test whether *mlcD* encodes an HMGCR, we conducted a complementation assay in *S. cerevisiae*. *S. cerevisiae* contains two genes encoding HMGCRs, called *HMG1* and *HMG2*, respectively. HMG1 was shown by Basson et al. to be responsible for approximately 83% of the HMGCR catalytic activity found in wild type cells (Basson et al., 1986a).

Firstly, we were interested in the effect that deletion of either *HMG1* or *HMG2* would have on yeast's statin sensitivity. We deleted the two genes in *S. cerevisiae* CEN.PK strain using the strategy described by Reid et al. (Reid et al., 2002b), resulting in the strains AR31 for the *HMG1* deletion and AR32 for the *HMG2*

deletion. The growth of the two strains on rich medium was not affected (Figure 2), results that are similar to those published by Basson et al. showing that cells containing a mutant allele of either *HMG1* or *HMG2* are viable, and have only a subtle growth effect (Basson et al., 1987, 1986a). However, when the strain sensitivity to statins was assayed, it showed that the AR31 strain was considerably more sensitive to extracellular lovastatin than the wild-type strain (Figure 2). The growth of the AR31 strain was completely inhibited already at the lowest tested lovastatin concentration (0.25 mM). In contrast, deletion of *HMG2* had very little effect on the lovastatin-sensitivity, as the AR32 strain behaves similarly to the wild-type strain on medium containing lovastatin (Figure 2). The hyper sensitivity of the *hmg1Δ* strain is in good agreement with the fact that HMG1 isozyme is responsible for the majority of the HMGCR activity in the cells (Basson et al., 1986b). Next, we investigated whether expression of *mlcD* in the *hmg1Δ* strain would rescue the observed statin hypersensitive phenotype. For this, *mlcD* was integrated into the two deletion strains as described in the previous section, resulting in strains AR33 (*hmg1Δ X3::PTEF1-mlcD*) and AR34 (*hmg2Δ X3::PTEF1-mlcD*). Testing of the two strains statin sensitivity levels showed that they behaved as the ARX4 strain (*X3::PTEF1-mlcD*) (Figure 2), indicating that *mlcD* was not only capable of complementing *hmg1Δ* mutation, but in addition made both strains less sensitive compared to the wild type reference. Lovastatin sensitivity of the constructed strains is summarized in Table 1.

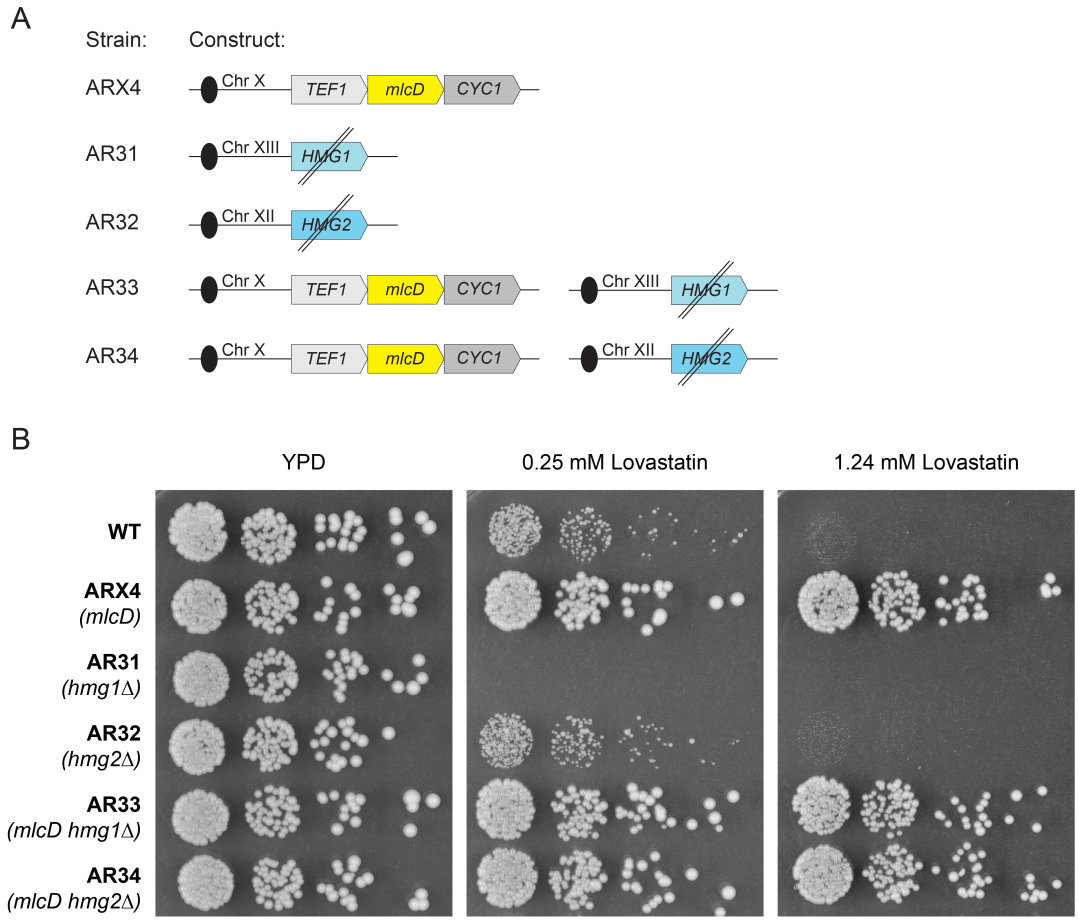

Figure 2: Susceptibility assay. (A) Overview of the constructed and tested strains. (B) Susceptibility assay: five-fold dilution series of the strains starting with and OD_{600} of 0.01 were prepared from overnight cultures and plated on a set of YPD agar plates containing activated lovastatin (0, 0.25 or 1.24 mM). The plates were incubated at 30°C for 3 days, after which the growth of the strains was recorded by photography. Strains: WT (CEN.PK113-11C); ARX4 (*X3::pTEF1-mlcD*); AR31 (*hmg1Δ*); AR32 (*hmg2Δ*); AR33 (*hmg1Δ X3::pTEF1-mlcD*); AR34 (*hmg2Δ X3::pTEF1-mlcD)*.

To determine whether MlcD is an HMGCR or mediates its statin resistance via an alternative activity (e.g. binding of stains) we performed a complementation experiment by deleted both HMGCR isozymes in *S. cerevisiae*. However, as the double deletion (*hmg1* and *hmg2)* has previously been shown to be nonviable (Basson et al., 1986a), we deleted the two genes in a strain expressing *mlcD* (strain ARX4). If the resulting strain was viable, then MlcD must be an HMGCR, as it would be the only enzyme present in the cells capable of reducing HMG-CoA to mevalonate and HMG-CoA. Deletion of both *HMG1* and *HMG2* in the *mlcD*-expressing strain (resulting strain is named ARX5) only had a minimal effect on the growth of the yeast cells on YPD plates compared to the wild-type strain (Figure 3),

echoing the results from Basson et al. that expression of either human or hamster HMGCR restored the viability of *hmg1 hmg2* yeast cells lacking this enzyme (Basson et al., 1988). The performed complementation experiment shows that MlcD is able to provide sufficient catalytic capacity to replace both HMGCR isozymes in yeast, confirming the hypothesis that *mlcD* encodes for an HMGCR. Moreover, the *mlcD*-expression setup in *S. cerevisiae* confers high-level lovastatin resistance even in the strain lacking both native HMGCRs (Figure 3).

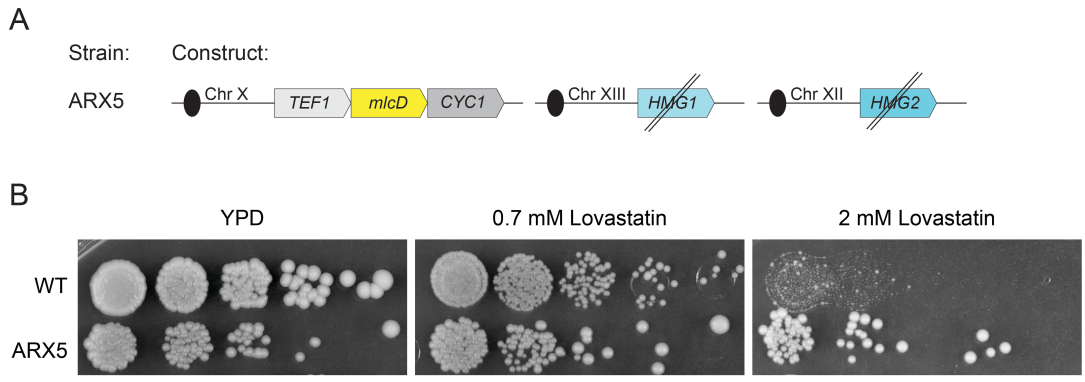

Figure 3: Susceptibility assay of the *S. cerevisiae* strain lacking native HMGCRs (*HMG1* and *HMG2*) and expressing *mlcD* (ARX5; *hmg1Δ hmg2Δ X3::pTEF1-mlcD*). (A) Schematic representation of the constructed strain. (B) Susceptibility assay. Ten-fold dilution series of the ARX5 and wild-type strain (WT; CEN.PK113-11C) strains starting with and OD_{600} of 0.02 were prepared from overnight cultures and plated on a set of YPD agar plates containing lovastatin (0, 0.7 or 2 mM). The plates were incubated at 30°C for 3 days, after which the growth of the strains was recorded by photography.

Table 1

Summary of the susceptibility assays.

Strain	Genotype	Statin sensitivity
CEN.PK113-11C (Wild type)	*MATα MAL2-8C SUC2 his3Δ ura3-52*	Reference
AR31	*MATα MAL2-8C SUC2 his3Δ ura3-52* ***hmg1Δ***	High
AR32	*MATα MAL2-8C SUC2 his3Δ ura3-52* ***hmg2Δ***	As reference
ARX4	*MATα MAL2-8C SUC2 his3Δ ura3-52* ***X3::pTEF1-mlcD***	Low
AR33	*MATα MAL2-8C SUC2 his3Δ ura3-52* ***hmg1Δ X3::pTEF1-mlcD***	Low
AR34	*MATα MAL2-8C SUC2 his3Δ ura3-52* ***hmg2Δ X3::pTEF1-mlcD***	Low
-	***hmg1Δ hmg2Δ***	Not viable
ARX5	*MATα MAL2-8C SUC2 his3Δ ura3-52* ***hmg1Δ hmg2Δ X3::pTEF1-mlcD***	Low

5.2.3 Do statin-producing fungi contain a unique HMGCR type?

We showed that MlcD is an HMGCR and that it can confer the resistance to statins, but what is the molecular basis for the statin resistance? We were interested whether the statin resistance is based on the overproduction of the statin target, which would suggest that statin producers have more than one HMGCR-encoding gene. Or do the statin producers contain a unique HMGCR, which is somehow resistant to statins, a situation similar to the presence of MPA-resistant IMPDH in *P. brevicompactum*? In the hope of gaining an insight into the molecular basis behind the resistance we used a bioinformatics approach.

5.2.3.1 Analysis of HMGCR copy number

The lovastatin and compactin gene clusters both include an HMGCR-encoding gene, however it is currently unknown how many HMGCRs fungi typically encode. To determine this, we performed a BlastP against the Join Genome Institute (JGI) fungal genome database (Nordberg et al., 2014) and the resulting hits were manually curated by eliminating doubtful gene models, missing data, and sequences, in which the conserved domain structure was not found (see section 5.4.4. for detailed description of the curating procedure). The resulting database included 583 HMGCR sequences, originating from 447 different genomes, and all the sequences included an 'HMG-CoA_red' (PF00368) domain, which is characteristic HMGCRs. To all the sequences in our database, information about the phyla/division was attributed, the redundancy was eliminated, e.g. species for which multiple different genomes were present such as *S. cerevisiae* (see section 5.4.5. for detailed description of the procedure), and the resulting dataset contained 551 unique sequences divided between 435 species from 319 different genera. We sorted the list based on species names and counted the number of putative HMGCR copies in the individual species (data not presented), showing that 82% of

the species across the entire taxonomic range encoded a single copy of the gene. The highest copy number found was 5, which were present in five species of ascomycetes and one of basidiomycetes. Sorting the list based on species name also revealed that our dataset is not taxonomically balanced as it includes enrichment for species within specific genera, for example twenty-one *Aspergillus* species. To compensate for this, a representative species (with respect to gene copy number) was selected from each genus. The final dataset was used for the copy-number count (Table 2). While this correction eliminated the 'multi-species per genera' problem, it did not remove the bias for Ascomycota and Basidiomycota[6]. The corrections only had a minor impact on the data and did not change the general trend with the majority (85%) of species containing a single copy of the HMGCR enzyme, and the highest copy number being 5 (Table 2).

Table 2

Number of putative HMGCRs per genome sorted by taxonomic phylum/division with a single representative for each genus.

Phylum/Division	Number of			Putative HMGCR copy-number				
	Genera	Species	Seq.	1	2	3	4	5
Ascomycota	178	178	219	153 (86.0%)	16 (9.0%)	4 (2.2%)	3 (1.7%)	2 (1.1%)
Basidiomycota	122	122	142	106 (86.9%)	14 (11.5%)	1 (0.8%)	-	1 (0.8%)
Zygomycota	12	12	22	5 (41.7%)	4 (33.3%)	3 (25.0%)	-	-
Blastocladiomycota	1	1	1	1 (100%)	-	-	-	-
Chytridiomycota	2	2	2	2 (100%)	-	-	-	-
Cryptomycota	1	1	1	1 (100%)	-	-	-	-
Glomeromycota	1	1	1	1 (100%)	-	-	-	-
Microsporidia	2	2	2	2 (100%)	-	-	-	-
Total	319	319	390	271 **(85.8%)**	34 (10.8%)	8 (2.5%)	3 (0.9%)	3 (0.9%)

5.2.3.2 Phylogenetic analysis of putative HMGCRs

[6] The bias is due to the available genome sequences where focus has been on ascomycetes and basidiomycetes.

Next, we performed a phylogenetic analysis of the putative HMGCRs. The dataset with 551 putative HMGCR sequences was aligned using MUSCLE (Edgar, 2004) to create higher quality alignments for the phylogenetic analysis. This showed that only the predicted 'HMG-CoA_red' region was shared between all the sequences. Unaligned regions were removed, only retaining the approximately 340 aa long 'HMG-CoA_red' domain in each sequence, which was used for the phylogenetic tree (Maximum Likelihood tree) construction. HMGCR from *Sulfolobus tokodaii* (Archaea)[7] was used as an out-group. Analysis of the resulting tree (Figure 4) showed that the majority of sequences grouped into five clades with good support (bootstrap values above 67)[8], which for the major part followed the taxonomy of the included species at the phylum level (Figure 4A). One significant exception from this grouping was that the sequences from ascomycetes were between two separate clades (Group A and Group B) with 100% bootstrap support.

The two clades proved to be very different (Figure 4B), with Group A sequences being characterized by containing three specific domains: (i) HPIH membrane spanning domain (PF13323.1; function unknown), (ii) sterol-sensing domain (PF12349.3) and (iii) 'HMG-CoA reductase' domain (PF00368.13) (Figure 5). In contrast, Group B sequences only contained an HMG-CoA reductase domain, similar to the situation in Cryptomycota, Microsporidia and Archaea HMGCRs (Figure 5). Group A (289 sequences) included representatives for all 266 analyzed ascomycete species (surplus of 22 sequences), while Group B (59 sequences) only included 35 different species. The domain structure and universal distribution of the HMGCRs in the Ascomycota Group A suggests that this is the ancestral HMGCR that is involved in central metabolism, catalyzing the conversion of HMG-CoA to mevalonate. The function of the previously unrecognized subgroup B is unknown, however the lack of transmembrane and sterol-sensing domains suggests that

[7] HMGCRs are divided into two classes, where class I includes eukaryotic and archaeal enzymes, while class II consists of enzymes from eubacteria (Bochar et al., 1999). Class II HMGCRs also catalyzes the reverse reaction of class I enzymes (Hedl et al., 2004).

[8] The phylogeny also included three additional smaller clades. Analysis of these clades revealed that they contained sequences form different phyla.

these HMGCRs are soluble and serve a different function than Group A. Though they share domain structure with the Cryptomycota and Achaea HMGCRs, Group B is as distantly related to these as it is to the Group A, and the origin of the Group B remains elusive[9]. The three HMGCRs known to be involved in statin resistance (MlcD, LvrA and MokG) were all found to belong to the clade Ascomycota Group A (Figure 4A).

[9] Requires dedicated study to be resolved – either BlastP against complete non-redundant database or by including additional groups of eukaryotes.

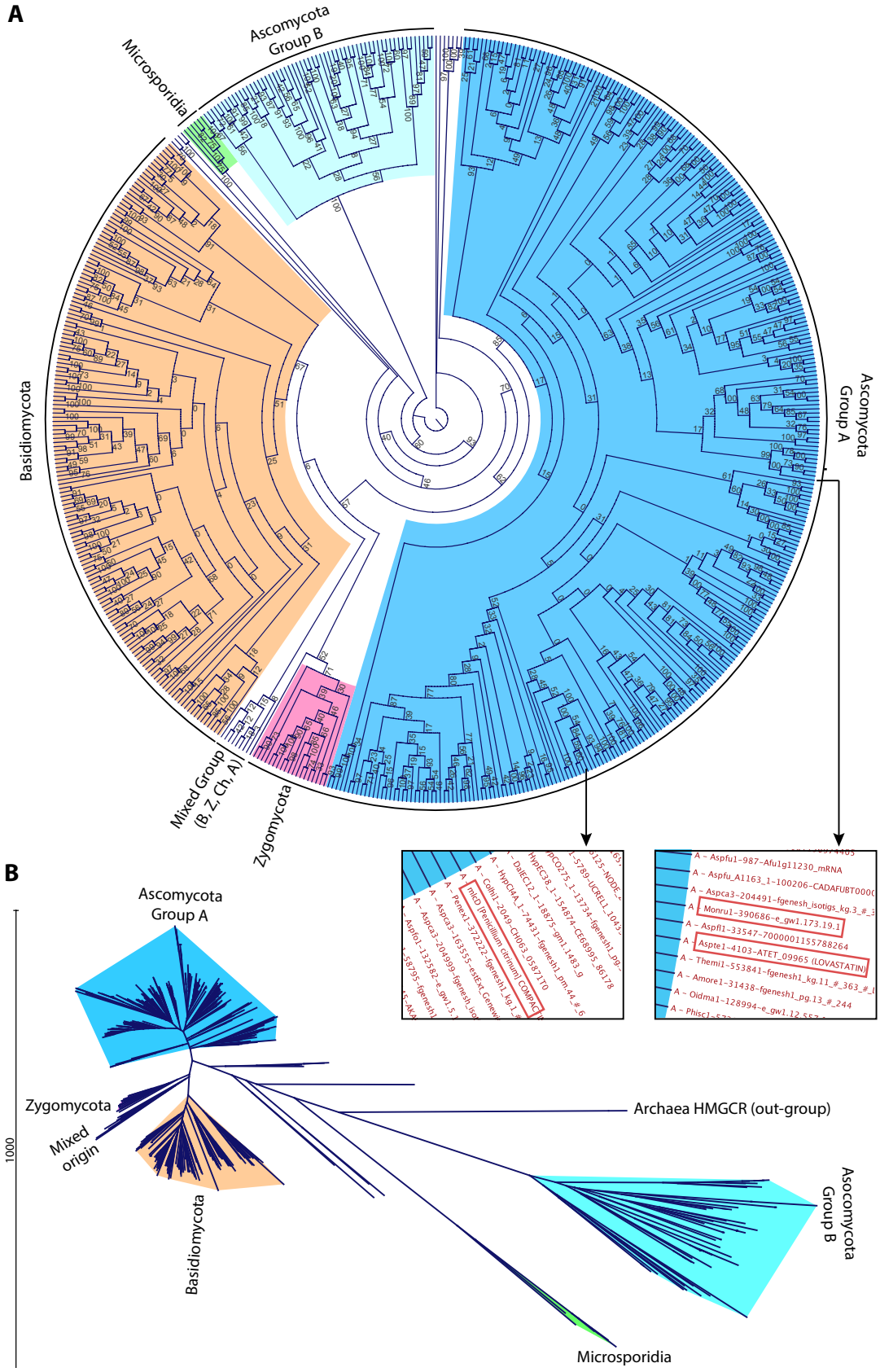

Figure 4: Phylogenetic analysis of putative HMGCRs. (A) Circular cladogram showing the maximum likelihood tree for the putative fungal HMGCRs with an Archaea HMGCR as outgroup. Arrows are indicating placement of MlcD (from *P. citrinum* compactin gene cluster; left quare) and LvrA and MokG (from *A. terreus* and *M. ruber* lovastatin gene cluster, respectively; right square) in the phylogeny. (B) Radial display of the phylogenetic tree shows the distances between the different groups. Abbreviations of the groups: B-basidiomycota, Z-zygomycota, A-ascomycota, Ch-chytridiomycota.

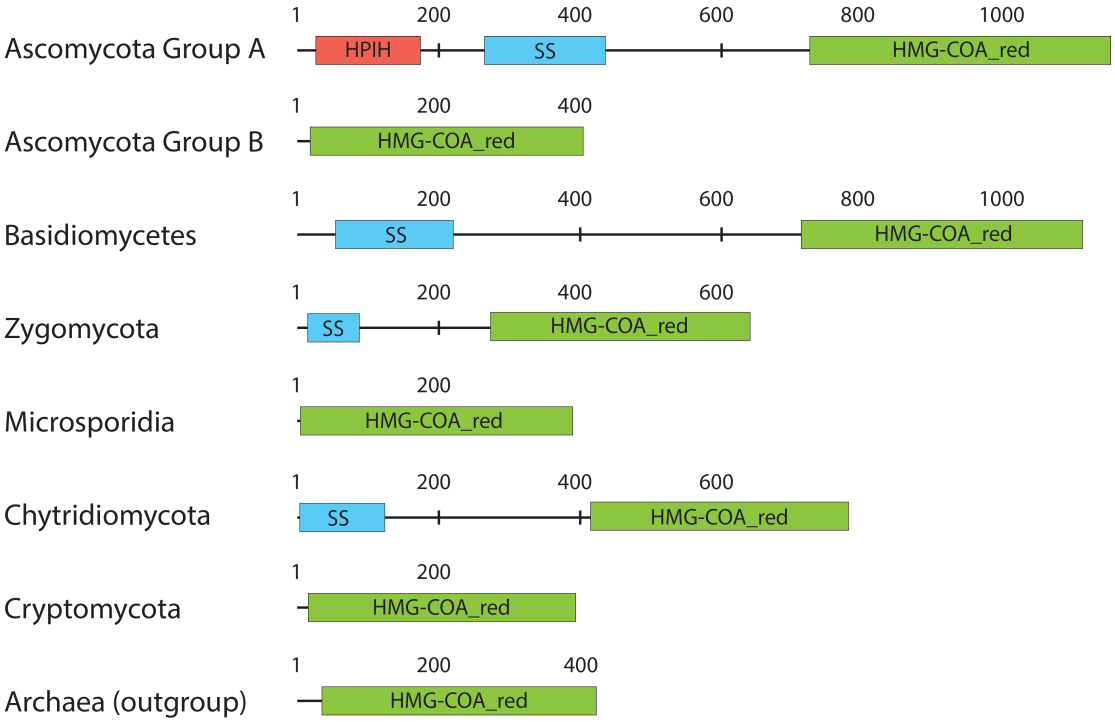

Figure 5: Domain architecture representative HMGCRs for the different clades highlighted in the phylogeny. Proteins are drawn to scale. The identified domains included 'HMG-CoA reductase' (HMG-COA_red; PF00368.13), HPIH (PF13323.1) and Sterol-sensing (SS; PF12349.3) domains. For the Microsporidia, Chytridiomycota and Cryptomycota groups only few sequences were available and it is unknown whether the included sequences are representative for the HMGCRs in these groups. Domains were identified using Pfam protein family database version 27.0 (Finn et al., 2014).

5.2.3.3 Analysis of the Ascomycota Group A HMGCRs

The clade Ascomycota Group A contains twenty-one species with two or more copies of the HMGCR from Group A, as shown in Table 3. Seventeen of these species belong to the *Asperillus* genus, revealing local enrichment for this enzyme class, supporting a model, where one or several gene duplication events have occured.

Table 3

Ascomycetes that possess more than one HMGCR-domain containing protein, sorted based on the number of enzymes belonging to Group A. Especially members of the *Aspergillus* genus (shown in blue) are enriched for HMGCRs belonging to Group A. Enrichment for group B HMGCRs is also seen.

	Number of HMGCRs		
Species	**in Group A**	**In Group B**	**Total**
Aspergillus carbonarius	**3**	1	4
Aspergillus flavus, Aspergillus oryzae	**2**	3	5
Aspergillus brasiliensis, Aspergillus acidus, Aspergillus kawachii, Aspergillus niger, Aspergillus phoenicis, Aspergillus terreus, Aspergillus tubingensis	**2**	2	4
Neosartorya fischeri	**2**	1	3
Aspergillus fumigatus , Aspergillus novofumigatus, Aspergillus sydowii, Aspergillus versicolor*, Monascus ruber, Penicillium expansum, Saccharomyces cerevisiae, Kazachstania africana, Lindgomyces ingoldianus, Colletotrichum eremochloae, Colletotrichum eremochloae*	**2**	0	2
Fusarium oxysporum	**1**	4	5
Corynespora cassiicola, Periconia macrospinosa	**1**	3	4
Aspergillus wentii*, Colletotrichum zoysiae, Myriangium duriaei, Penicillium bilaiae*	**1**	2	3
Penicillium brevicompactum, Penicillium chrysogenum, ***Aspergillus glaucus, Aspergillus nidulans, Aspergillus steynii,*** *Aureobasidium pullulans Byssothecium circinans, Colletotrichum higginsianum, Colletotrichum somersetensis, Glonium stellatum, Ilyonectria sp., Marssonina brunnea, Mytilinidion resinicola, Niesslia exilis, Penicillium glabrum, Phialocephala scopiformis, Pyrenochaeta sp., Stanjemonium grisellum*	**1**	1	2

To further investigate the genera that contain statin-producing species, a MUSCLE alignment and Maximum Likelihood tree were calculated for members of the *Aspergillus*, *Penicillium* and *Monascus* genera. The resulting phylogeny (Figure 6) includes a subgroup (termed Primary HMGCR in the figure) supported by a bootstrap value of 100, which contains HMGCR sequences from all the included species. Though parts of the subtree topology (*Aspergillus* clade) did not reach significant support, *Aspergillus, Monascus* and *Penicillium* sequences (shown in

blue) were separated with bootstrap values of 84 and 100. This clade (Primary HMGCR) likely represents the HMGCR's that participate in primary metabolism. A second subgroup was separated from the 'Primary HMGCR' clade with a bootstrap support of 100. This group included the LvrA and MokG sequences from the lovastatin clusters in *A. terreus* and *M. rubrum,* respectively, and a second copy of HMGCR from *A. flavus* and *A. carbonarius*. The MlcD protein is found in a third clade where it clusters together with a second HMGCR from *Penicillium expansum* with a 100% support. The split of the LvrA/MokG and MlcD HMGCRs into two different clades could suggest that they have evolved by two independent events (separate duplication events; further discussed in the next section). Though *P. expansum, A. flavus* and *A. carbonarius* possess HMGCRs that cluster together with HMGCRs associated with statin-gene clusters, production of statins by these organisms have not been reported to our knowledge.

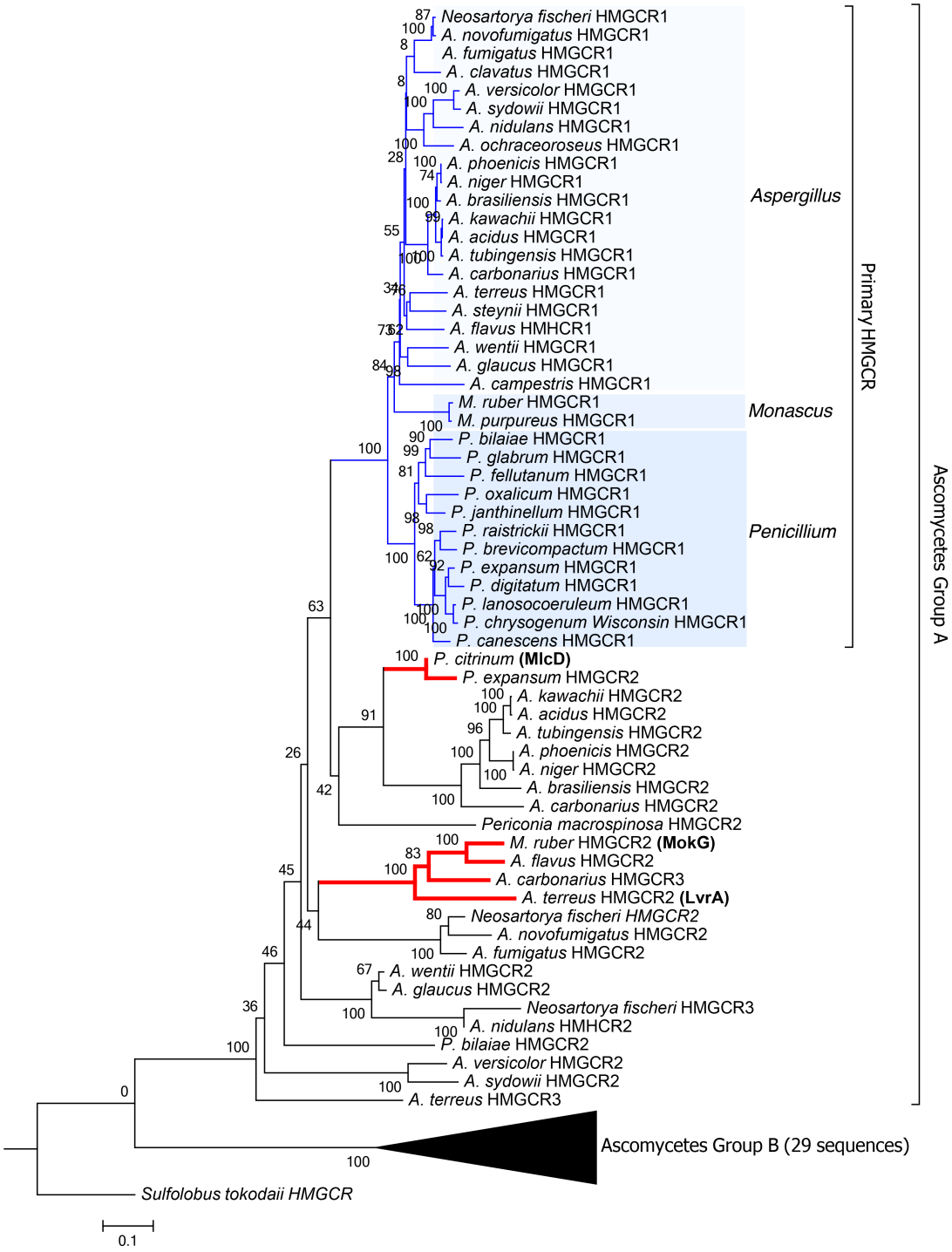

Figure 6: Phylogenetic analysis of the Ascomycota Group A HMGCRs. Subtree of the maximum likelihood tree for putative HMGCRs from the *Aspergillus* (including *Neosartorya*), *Monascus* and *Penicillium* genera, showing sequences from the Ascomycetes Group A. **Blue** color represents the "primary HMGCRs" and **red** color represents clades where HMGCRs from the statin gene clusters can be found (highlighted with bold font). Detailed list of sequences used for the analysis is shown in Table 6.

5.2.3.4 Gene duplication and evolving a new function

Gene duplication events result in a functional/genetic redundancy, which is an inherently unstable situation in an evolutionary perspective (Hartman et al., 2001). This can either be resolved by eliminating one of the genes or by one of the genes coming to serve a new function (catalytic or expression pattern). As the HMGCRs from the statin gene clusters do not cluster together with the primary HMGCRs found in the producing species (Figure 6), but with separate clades, they are likely not the result of recent duplication of the primary HMGCR. In addition, the tree presented in Figure 6 suggests that *mlcD* and *lvrA*/*mokG* have arisen from separate duplication events. The genetic redundancy has likely allowed for the adaptation to serve a new function in protecting the cell from the toxic effects of statin. We propose that the HMGCR-encoding gene at some time during evolution was duplicated, and genome shuffling has resulted in relocation of the gene into the gene clusters. The selective advantage of this genetic setup was then preserved throughout time/evolution as it posed a selective advantage.

If HMGCRs encoded in the statin gene clusters arose from the recruitment of the gene into the gene cluster by a gene duplication event, then the statin self-resistance mechanism is likely based on the increased concentration of HMGCR in the cell at the time when statins are being synthesized. Alternatively, HMGCRs from the statin gene clusters are unique versions of the enzyme that developed during evolution in a way to become insensitive to statins. It has been previously shown that some of the amino acid residues in human HMGCR are associated with statin sensitivity of the enzyme; i.e. Q766H mutation incresed statin sensitivity of the enzyme (Wysocka-Kapcinska et al., 2009). To look for possible substitutions in HMGCRs associated with the statin clusters, which might indicate their statin insensitivity, a protein sequence alignement of their catalytic region (HMG-COA_red; PF00368.13) was created using Clustal Omega program (Sievers et al., 2011) (Figure 7). Human HMGCR was used as a reference to identify the residues that participate

in binding of HMG-CoA (the residues were defined experimetally (Istvan, 2000)), and that were previously suggested to be associated with statin sensitivity (i.e. position 766).

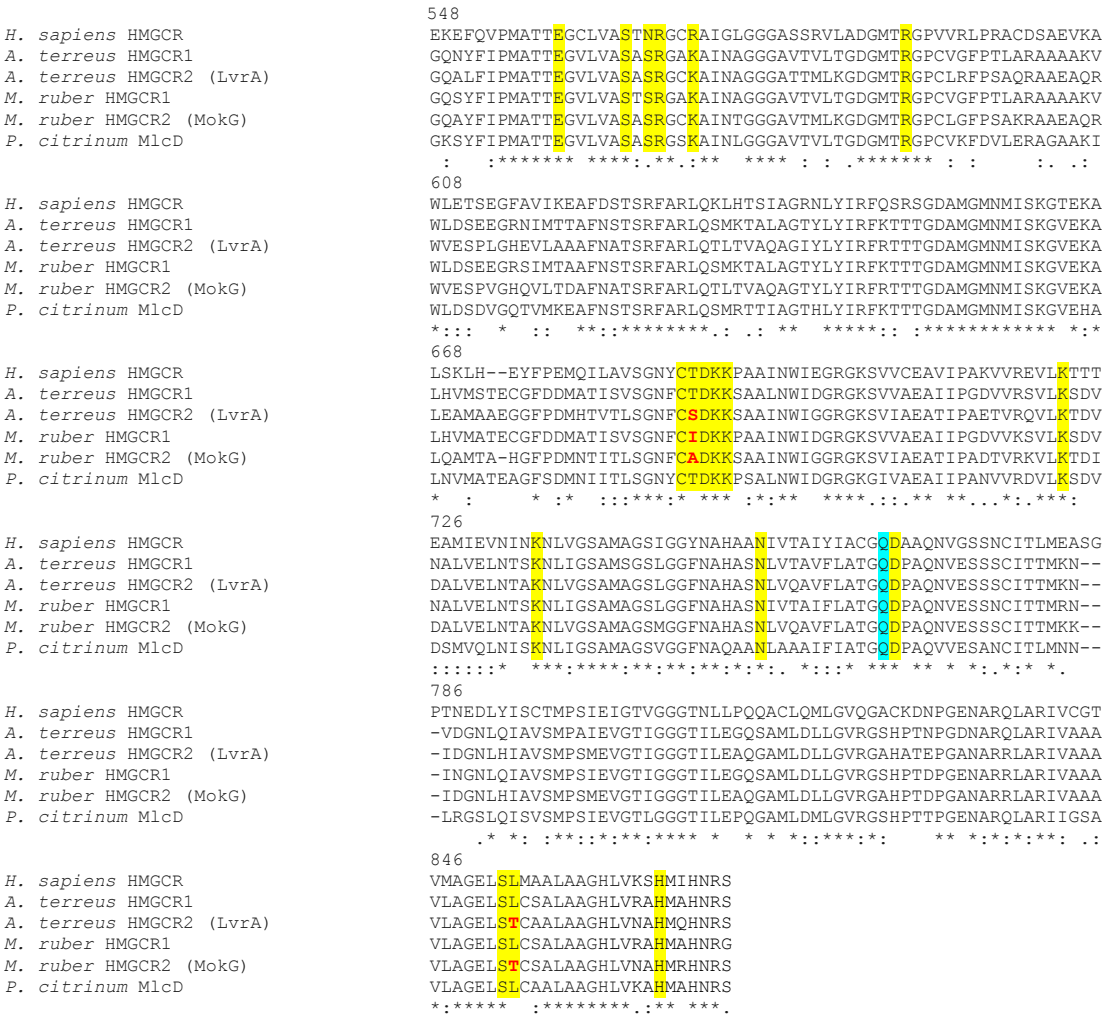

Figure 7: Sequence alignment of the catalytic domains of HMG-CoA reductases (HMGCRs). The region including the residues that participate in the binding of HMG-CoA is presented in the figure. The numbers on top of the alignment correspond to the amino acid positions in human HMGCR (*Homo sapiens* HMGCR). The HMG-CoA-binding residues in human HMGCR (marked with **yellow**) were identified prior to this work (Istvan, 2000). Position 766 that was prveviously shown to be important for statin sensitivity is marked witjh **blue** (Wysocka-Kapcinska et al., 2009). Non-conserved residues are shown in **red**. Sequences were aligned with Clustal Omega program (Sievers et al., 2011). Symbols below the alignment: * (asterisk)-positions, which have a single, fully conserved residue. : (colon)-conservation between groups of strongly similar properties (scoring > 0.5 in the Gonnet PAM 250 matrix) . (period)-conservation between groups of weakly similar properties (scoring =< 0.5 in the Gonnet PAM 250 matrix).

Sequence comparison revealed that the residue at position 766 (shown in blue in Figure 7), which was proposed to be associated with the enzyme`s statin sensitivity (Wysocka-Kapcinska et al., 2009), is conserved in all the aligned HMGCR sequences. Most of the residues that participate in the binding of HMG-CoA, and

thus also statins (shown in yellow in Figure 7), are too conserved, however, few residues (shown as red in Figure 7) in *A. terreus* LvrA, and in *M. ruber* HMHCRs are substituted, suggesting that these HMGCRs could possess altered binding affinity for statins. If these substitutions would render the HMGCRs stain-insensitive, than one would expect that the substitutions would be also present in MlcD. The fact that not all the statin-associated HMGCRs possess similar substitutions, combined with the finding that MlcD and LvrA/MokG clades arose from separate duplication events, favor the hypothesis that the statin self-resistance mechanism is based on the increased concentration of HMGCR and not on the expression of the statin-insensitive version of HMGCR. Nevertheless, the question whether the HMGCRs encoded in the statin gene clusters are statin insensitive can only be resolved via an *in vitro* biochemical characterization of the HMGCRs from the statin-clusters.

5.3 Conclusions

This study shows that *mlcD* from the *P. citrinum* compactin gene cluster can mediate statin resistance when expressed heterologously in *S. cerevisiae*, supporting the suggestions made by Alberts *et al.* concerning the function of the orthologues function in *A. terreus*. Successful complementation of HMG1 and HMG2 in yeast, in addition, for the first time provides direct evidence for the mechanism by which MlcD mediates resistance, as it functions as HMGCR. With the ability to confer the statin resistance in *S. cerevisiae*, MlcD has a potential to improve future yeast based production of statins. To gain insight into the molecular biological basis of statin resistance, a bioinformatics approach was used. Our phylogenetic analysis showed that HMGCRs from the statin clusters split between two different clades (MlcA in one clade and LvrA/MokA in another clade), which could suggest that they have evolved by two independent events (separate duplication events). HMGCR-encoding gene at some time during evolution was likely duplicated, and genome shuffling has resulted in relocation of the gene into

the statin gene clusters. Moreover, protein sequence alignment did not reveal any unique amino acid substitutions, which would suggest that the HMGCRs from the statin gene clusters are statin-insensitive. We propose that the statin self-resistance mechanism is based on the increased concentration of HMGCR in the cell and not on the expression of the statin-insensitive version of HMGCR, however, further biochemical characterization of the HMGCRs from the statin-clusters is required to validate the proposed model. Understanding the molecular biological basis for statin resistance may aid the strain optimization processes for efficient production of statins in both, native and heterologous hosts.

5.4 Materials and methods

5.4.1 Construction of plasmids and strains

A yeast codon-optimized version of the *mlcD* gene, *de novo* synthetized by Genscript, was PCR amplified from the plasmid pEN668 with primers mlcD-F and mlcD-R. The *S. cerevisiae TEF1* promoter was amplified from the plasmid pSP-G2 (Partow et al., 2010) using primers TEF1-d and PGK1-s. The amplified fragments were cloned into the pX-3 targeting vector (Mikkelsen et al., 2012) via the USER cloning technique (Nour-Eldin et al., 2006) resulting in plasmid pX3-TEF1-mlcD-CYC1. All fragments were amplified by PCR using a USER cloning compatible PfuX7 polymerase (Nørholm, 2010). *Escherichia coli* DH5α (Woodcock et al., 1989) was used as host for USER cloning experiments and for the propagation of the constructed plasmid. The inserts of the resulting plasmid were verified by sequencing (StarSEQ). The constructed plasmid was digested with the NotI enzyme (New England Biolabs), and the obtained linear fragment was used for yeast transformation using the lithium acetate/single-stranded carrier DNA/polyethylene glycol transformation method (Gietz and Schiestl, 2007). The linear gene targeting cassette was integrated into the X-3 locus of the reference yeast strain, *S. cerevisiae* CEN.PK 113-11C as described by Mikkelsen et al (Mikkelsen et al., 2012). Correct integration of the substrate was verified by diagnostic colony PCR with one primer annealing outside of the integration site in the yeast genome (X-3-up-out-sq), and one substrate specific primer (C1_TADH1_F).

For the deletion of the HMGCR-encoding genes in *S. cerevisiae* we used a gene targeting strategy (Reid et al., 2002a, 2002b), in which the targeted genes (*HMG1* and *HMG2*) were replaced by the *Kluyveromyces lactis URA3* marker that was flanked by a direct repeat allowing for iterative gene targeting (Figure 8). Four PCR fragments for each knockout were generated. Two fragments represented the upstream and downstream regions of the gene to be deleted (HMG1/HMG2

upstream and HMG1/HMG2 downstream, respectively), and two fragments represented the 5` 2/3 of *K. lactis URA3* and the 3` 2/3 of *K. lactis URA3* marker (URA 1 and URA2 respectively). The URA1 and URA2 fragments were amplified from plasmid pWJ1042 (Reid et al., 2002a, 2002b) using primer pairs 5`-int/cKL3`and 3`-int/dKL5`, respectively. The HMG1 upstream and HMG1 downstream fragments were amplified from genomic DNA of the WT strain using primer pairs HMG1-Up-Fv/HMG1-Up-Rv and HMG1-Dw-Fv/HMG1-Dw-Rv, respectively, and the HMG2 fragments were obtained in the same way using the according primers. Next, gene-targeting substrates were generated by PCR-mediated fusion, in which HMG1 upstream fragment was fused with URA2, and HMG1 downstream with URA1. Similarly, HMG2 upstream and HMG2 downstream fragments were fused with URA2 and URA1 fragments, respectively. This resulted in four gene targeting substrates, which were used for yeast transformation using the lithium acetate/single-stranded carrier DNA/polyethylene glycol transformation method (Gietz and Schiestl, 2007). Correct integration of substrates was verified by diagnostic colony PCR. Primer pairs HMG1-Up-out/HMG1-Dw-out and HMG2-Up-out/HMG2-Dw-out were used to confirm the deletion of the *HMG1* and *HMG2* gene, respectively.

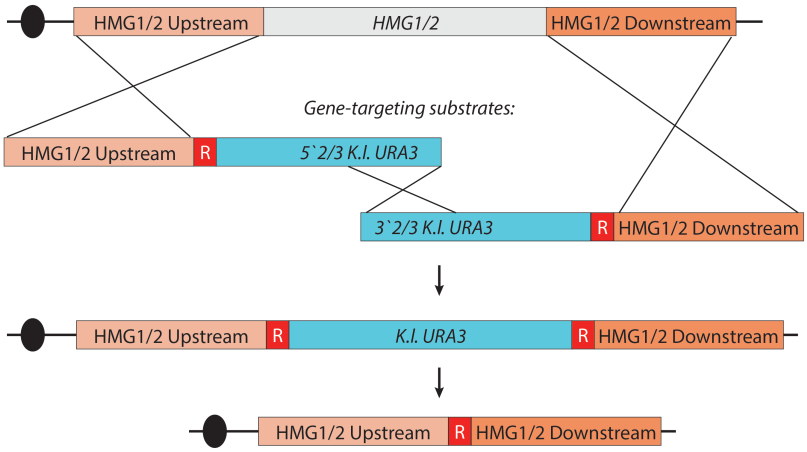

Figure 8: Schematic representation of the gene targeting strategy used to delete the HMGCR-encoding genes, *HMG1* and *HMG2*, in *S. cerevisiae*. The targeting substrates, each composed of the targeting sequence (HMG1/2 upstream or HMG1/2 downstream) and 2/3 of *K. lactic URA3* marker, were used to replace the targeted genes with the marker via homologous recombination (indicated with crosses). The *URA3* marker was flanked by direct repeats, enabling removal of the marker by direct repeat recombination.

The *URA3* markers in all the constructed strains were removed by direct repeat recombination using 5-FOA (Melford) counter selection. Oligonucleotides used in this study are listed in Table 4, and plasmids and strains are listed in Table 5.

Table 4

Oligonucleotides used in this study. U = 2-deoxyuridine.

Primer name	Primer sequence (5`- 3`)	Use
mlcD-F	ATCAACGGGUAAAAATGGTGGCTTCCT	Amplification of *mlcE* from plasmid pEN669
mlcD-R	CGTGCGAUTCAACGTCTGGCAC	
TEF1-d	ACGTATCGCUGTGAGTCGTATTACGGATCCTTG	Amplification of promoter sequence from plasmid pSP-G2
PGK1-s	CGTGCGAUGCCGCTTGTTTTATATTTGTTG	
X-3-up-out-sq	TGACGAATCGTTAGGCACAG	Strain confirmation via colony PCR
C1_TADH1_F	CTTGAGTAACTCTTTCCTGTA	
cKL3`	CACGGCGCGCCTAGCAGCGGTAACGCCAGGGTTTTCCCAGTCAC	Amplification of URA1 fragment from plasmid pWJ1042
5`-int	CTTGACGTTCGTTCGACTGATGAGC	
3`-int	GAGCAATGAACCCAATAACGAAATC	Amplification of URA2 fragment from plasmid pWJ1042
dKL5	GTCAGCGGCCGCATCCCTGCTTCGGCTTCATGGCAATTCCCG	
HMH1-Up-Fv	GAAACTTTTTGGTCGGTC	Amplification of HMG1 upstream fragment from gDNA of WT strain
HMG1-Up-Rv	GCAGGGATGCGGCCGCTGACCCTAAACTTAGTCATACGTC	
HMH1-Dw-Fv	CCGCTGCTAGGCGCGCCGTGCGGCATGCTTGTTTTATG	Amplification of HMG1 downstream fragment from gDNA of WT strain
HMG1-Dw-Rv	AAACGGAATTTCAAAGGGC	
HMH2-Up-Fv	TATTTAGTGATATAGCCGCCCA	Amplification of HMG2 upstream fragment from gDNA of WT strain
HMG2-Up-Rv	GCAGGGATGCGGCCGCTGACGAAGTGACATTTGAGGTTG	
HMH2-Dw-Fv	CCGCTGCTAGGCGCGCCGTGCCCTGTAAAACCTCAGCATTAT	Amplification of HMG2 downstream fragment from gDNA of WT strain
HMG2-Dw-Rv	GGACATTCTTTTTAGCACAC	
HMG1-Up-out	TTGTGTGGCCTTATCTATGC	Strain confirmation via colony PCR
HMG1-Dw-out	TTCTCTTCCAACCCCTTTTAC	
HMG2-Up-Out	TCCATGAAATAGGGTCCTT	Strain confirmation via colony PCR
HMG2-Dw-Out	TCTCGGTTCTATTTCTGTCGT	

Table 5

Plasmids and strains used in this study.

Plasmid name	Description	Reference or source
pEN668	Template for amplifying *mlcD* (*S. cerevisiae* codon optimized)	from Evolva Holding SA
pWJ1042	Template for amplifying *URA3* marker	(Reid et al., 2002b)
pSP-G2	Template for amplifying *TEF1*	(Partow et al., 2010)
pX3	USER cloning vector equipped with the *CYC1* terminator designed to target site 3 on chromosome X.	(Mikkelsen et al., 2012)
pX3-TEF1-mlcD-CYC1	Plasmid carrying a gene-targeting cassette for expressing *mlcD* in yeast.	This study
Strain name	**Genotype**	**Reference or source**
Escherichia coli		
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	(Woodcock et al., 1989)
Saccharomyces cerevisiae		
CEN.PK113-11C (Wild type strain)	*MATα MAL2-8C SUC2 his3Δ ura3-52*	Dr. Petter Kötter, Institut für Mikrobiologie, der Johan Wolfgang Goethe-Universität, Frankfurt am Main, Germany
ARX4	*MATα MAL2-8C SUC2 his3Δ ura3-52 X3::PTEF1-mlcD-Tcyc1*	This study
AR31	*MATα MAL2-8C SUC2 his3Δ ura3-52 hmg1Δ*	This study
AR32	*MATα MAL2-8C SUC2 his3Δ ura3-52 hmg2Δ*	This study
AR33	*MATα MAL2-8C SUC2 his3Δ ura3-52 hmg1Δ X3::PTEF1-mlcD-Tcyc1*	This study
AR34	*MATα MAL2-8C SUC2 his3Δ ura3-52 hmg2Δ X3::PTEF1-mlcD-Tcyc1*	This study
ARX5	*MATα MAL2-8C SUC2 his3Δ ura3-52 hmg1Δ hmg2Δ X3::PTEF1-mlcD-Tcyc1*	This study

5.4.2 Media

The *E. coli* transformants were selected on lysogeny broth (LB) medium containing 100 µg/mL of ampicillin. Yeast strains were cultivated in standard liquid or solid yeast peptone dextrose medium (YPD), synthetic complete medium (SC), or synthetic medium (SM). SC medium was prepared according to Sherman et al. (Sherman et al., 1986), with the minor modification that the L-leucine concentration was doubled to 60 mg/L. Yeast transformants were selected on SC medium lacking uracil. Removal of the *URA3* marker, via direct repeat recombination, was achieved

by growing the strain on SC medium containing 5-fluororotic acid (5-FOA; 740 mg/L, Melford) and uracil (30 mg/L).

For susceptibility experiments strains were grown aerobically on YPD plates, supplemented with activated lovastatin (Tokyo Chemical Industry). Lovastatin stock solution (50 mM) was prepared as described previously (Morimoto et al., 2013). Briefly, solid lovastatin was dissolved in 1 mL of 99% ethanol, preheated to 50°C, alkalinized with 0.5 mL of 0.6 M NaOH and incubated at 50°C for 2 hours. The pH of the solution was then adjusted to 7.2 by adding 0.4 M HCl. The final volume of the solution was adjusted to 2 mL with water, resulting in stock solutions of 50 mM. All stock solutions were filter-sterilized and stored at -20°C.

5.4.3 Susceptibility assays

For susceptibility assays on solid media five- or tenfold dilution series of *S. cerevisiae* strains, starting with an OD_{600} of 0.01 or 0.02 were prepared from overnight cultures in YPD medium (30°C/150 rpm). 4.5 µL of each dilution were plated on a set of YPD agar plates containing different cytotoxic compounds. The plates were incubated at 30°C for 3 days, after which the growth of the yeast strains was recorded by photography.

5.4.4 Identification of HMGCR candidates

To determine the number of HMGCR-encoding genes across fungal genomes we performed a BlastP against the JGI Genome Portal (Nordberg et al., 2014), which resulted in 649 hits, all with an E-value below e-7 and a bit score higher than 148, originating from 447 different genomes belonging to 346 different genera. The full-length sequences of the hits were retrieved and the sequences were manually curated to identify doubtful gene models and missing data. This was done by tentatively aligning the putative HMGCRs using the CLC Main Workbench

alignment algorithm and manually removing sequences with large stretches of missing information (gaps) in parts that were found in 95% of the other sequences. The eliminated sequences likely included both true pseudogenes and low quality gene models, however, no further effort was made to improve or test the validity of the underlying gene models. The remaining 602 amino acid sequences were analyzed for the presence of conserved domains using the build-in Pfam (Finn et al., 2014) annotation tool in CLC Main Workbench. Following the Pfam analysis the dataset was tentatively aligned again and clustered using the 'RapidNJ tree' tool in CLC Main Workbench, to allow for manual identification and elimination of sequences that did not show the same domain structure as closely related sequences. This reduced the total number of sequence hits to 583 representing 435 different genomes. All the retained sequences included a 'HMG-CoA_red' (PF00368) domain characteristic of HMGCRs.

5.4.5 Analysis of the HMGCR copy-number in fungal genomes

Complete lineages for the individual hits were retrieved from the National Center for Biotechnology Information (NCBI) taxonomy database (Federhen, 2002) and information about the phyla/division (ascomycota (A), basidiomycota (B), blastocladiomycota (Bl), chytridiomycota (Ch), cryptomycota (Cy), glomeromycota (G), microsporidia (M) and zygomycota (Z)) for the individual sequence belonged to was added to the sequence name. Using Microsoft Excel the putative HMGCRs were sorted based on first 'division name', secondly 'genera name' and thirdly 'species name'. The list was inspected and redundancy was eliminated, e.g. species for which multiple different genomes were present such as *S. cerevisiae*, resulting in a dataset containing 551 unique sequences divided between 435 species from 319 different genera. Sorting of the sequence list based on species names allowed for counting the number of putative HMGCR copies in the individual species.

5.4.6 Phylogenetic analysis of putative HMGCRs and Ascomycota Group A HMGCRs

The dataset with 551 putative HMGCR sequences was aligned using MUSCLE (Edgar, 2004) to create higher quality alignments for the phylogenetic analysis. The sequences fell within three groups based on sequence length and predicted domain structure, which was reflected in the MUSCLE alignments where only the predicted 'HMG-CoA_red' region was shared between all sequences. Unaligned regions were removed, only retaining the approximately 340 aa long 'HMG-CoA_red' domain in each sequence. To allow for rooting of the constructed phylogentic tree, the sole HMGCR from *Sulfolobus tokodaii* (Archaea) was included in the dataset to serve as an out-group. The sequences were realigned with MUSCLE and a Maximum likelihood phylogeny was built using MEGA 6 (Tamura et al., 2013). The latter approach was used for generation of phylogenetic trees in both, Figure 4 and Figure 6.

5.4.7 Protein sequence alignment

Protein sequences were obtained from either JGI Genome Portal or NCBI. Reference numbres of the aligned fungal proteins are listed in Table 6. Human HMGCR was used as a reference (*H. sapiens* HMGCR; NCBI Accession NP_000850). Protein sequences were aligned with Clustal Omega program (Sievers et al., 2011) using the default settings.

Table 6

List of protein sequence used for the phylogenetic analysis of the Ascomycota Group A HMGCRs (presented in Figure 6). Sequences are listed in the same order as they appear in the phylogenetic tree from top to bottom. Where Protein ID from JGI is not available, an NCBI GenBank number (*), or Reference Sequence number (**) is provided.

Organism	Protein name used in the tree	JGI Protein ID
Neosartorya fischeri NRRL 181	HMGCR1	1520
Aspergillus novofumigatus IBT 16806 v1.0	HMGCR1	456029
Aspergillus fumigatus A1163	HMGCR1	100206
Aspergillus clavatus NRRL 1	HMGCR1	3868
Aspergillus versicolor v1.0	HMGCR1	132429
Aspergillus sydowii v1.0	HMGCR1	149226
Aspergillus nidulans from aspGD	HMGCR1	2327
Aspergillus ochraceoroseus IBT 24754	HMGCR1	513848
Aspergillus phoenicis ATCC 13157 v1.0	HMGCR1	133230
Aspergillus niger CBS 513.88	HMGCR1	4864
Aspergillus brasiliensis	HMGCR1	123745
Aspergillus kawachii IFO 4308	HMGCR1	15042
Aspergillus acidus v1.0	HMGCR1	141251
Aspergillus tubingensis v1.0	HMGCR1	125382
Aspergillus carbonarius ITEM 5010 v3	HMGCR1	207403
Aspergillus terreus NIH 2624	HMGCR1	3658
Aspergillus steynii IBT 23096 v1.0	HMGCR1	364578
Aspergillus flavus NRRL3357	HMGCR1	27661
Aspergillus wentii v1.0	HMGCR1	51536
Aspergillus glaucus v1.0	HMGCR1	119821
Aspergillus campestris IBT 28561 v1.0	HMGCR1	316459
Monascus ruber NRRL 1597 v1.0	HMGCR1	389114
Monascus Purpureus v1.0	HMGCR1	512502
Penicillium bilaiae ATCC 20851 v1.0	HMGCR1	392097
Penicillium glabrum DAOM 239074 v1.0	HMGCR1	385979
Penicillium fellutanum ATCC 48694 v1.0	HMGCR1	350461
Penicillium oxalicum 114-2	HMGCR1	5304
Penicillium janthinellum ATCC 10455 v1.0	HMGCR1	373341
Penicillium raistrickii ATCC 10490 v1.0	HMGCR1	315658
Penicillium Brevicompactum v2.0	HMGCR1	41253

Penicillium expansum ATCC 24692 v1.0	HMGCR1	380985
Penicillium digitatum PHI26	HMGCR1	5769
Penicillium lanosocoeruleum ATCC 48919 v1.0	HMGCR1	368062
Penicillium chrysogenum Wisconsin 54-1255	HMGCR1	142996
Penicillium canescens ATCC 10419 v1.0	HMGCR1	364500
Penicillium citrinum	MlcD	BAC20567.1*
Penicillium expansum ATCC 24692 v1.0	HMGCR2	372222
Aspergillus kawachii IFO 4308	HMGCR2	12145
Aspergillus acidus v1.0	HMGCR2	132582
Aspergillus tubingensis v1.0	HMGCR2	58795
Aspergillus phoenicis ATCC 13157 v1.0	HMGCR2	280144
Aspergillus niger NRRL3	HMGCR2	8211
Aspergillus brasiliensis v1.0	HMGCR2	44387
Aspergillus carbonarius ITEM 5010 v3	HMGCR2	163555
Periconia macrospinosa DSE2036 v1.0	HMGCR1	28317
Monascus ruber NRRL 1597 v1.0	HMGCR2	390686
Aspergillus flavus NRRL3357	HMGCR2	33547
Aspergillus carbonarius ITEM 5010 v3	HMGCR3	204491
Aspergillus terreus NIH 2624	HMGCR2	4103
Neosartorya fischeri NRRL 181	HMGCR2	2813
Aspergillus novofumigatus IBT 16806 v1.0	HMGCR2	504007
Aspergillus fumigatus A1163	HMGCR2	101185
Aspergillus wentii v1.0	HMGCR2	119346
Aspergillus glaucus v1.0	HMGCR2	129472
Neosartorya fischeri NRRL 181	HMGCR3	2369
Aspergillus nidulans from aspGD	HMGCR2	7253
Penicillium bilaiae ATCC 20851 v1.0	HMGCR2	391090
Aspergillus versicolor v1.0	HMGCR2	38907
Aspergillus sydowii v1.0	HMGCR2	194492
Aspergillus terreus NIH 2624	HMGCR3	5708
Sulfolobus tokodaii	HMGCR	WP_010979393.1**

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CHAPTER 6 (Article)

Heterologous expression of MlcE in *Saccharomyces cerevisiae* provides resistance to natural and semi-synthetic statins

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Authors:

Ana Ley[a], Hilde Cornelijne Coumou[a], Rasmus John Normand Frandsen [a]

[a]Department of Systems Biology**,** Technical University of Denmark, Søltofts Plads 223, 2800 Kgs. Lyngby, Denmark

Abstract

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the key enzyme in cholesterol biosynthesis. Their extensive use in treatment and prevention of cardiovascular diseases placed statins among the best selling drugs. Construction of *Saccharomyces cerevisiae* cell factory for the production of high concentrations of natural statins will require establishment of a non-destructive self-resistance mechanism to overcome the undesirable growth inhibition effects of statins. To establish active export of statins from yeast, and thereby detoxification, we integrated a putative efflux pump-encoding gene *mlcE* from the mevastatin-producing *Penicillium citrinum* into the *S. cerevisiae* genome. The resulting strain showed increased resistance to both natural statins (mevastatin and lovastatin) and semi-synthetic statin (simvastatin) when compared to the wild type strain. Expression of RFP-tagged *mlcE* showed that MlcE is localized to the yeast plasma and vacuolar membranes. We provide a possible engineering strategy for improvement of future yeast based production of natural and semi-synthetic statins.

Keywords:

Polyketide; Statins; *Saccharomyces cerevisiae*; Transport; Cell factory; Resistance

6.1 Introduction

Statins are used as cholesterol-lowering drugs in treatment and prevention of coronary heart diseases, and their extensive worldwide usage placed them among the best selling pharmaceuticals in the past decade (GBI Research, 2013). The application of statins in medicine is based on their ability to inhibit the catalytic action of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR). HMGCR constitutes the rate-limiting enzyme in the mevalonate pathway, which is responsible for the production of sterols, such as cholesterol in animal cells, and ergosterol in fungi (Maury et al., 2005). Natural statins are synthesized as secondary metabolites by filamentous fungi; mevastatin (Fig. 1A) by *Penicillium citrinum* (Endo et al., 1976a), and lovastatin (Fig. 1B) by *Aspergillus terreus* (Alberts et al., 1980) and *Monascus ruber* (Endo, 1979). Industrial scale production of natural statins and their semi-synthetic derivatives (e.g. simvastatin and pravastatin) is based on fermentation of statin-producing filamentous fungi (Manzoni and Rollini, 2002; S. K. Singh and Pandey, 2013). Production limitations associated with the unique physiology and morphology of these natural producers can be overcome by heterologous expression of the biosynthetic pathway in a fast-growing host, such as *Saccharomyces cerevisiae*. It will, however be crucial to establish a nondestructive resistance mechanism in yeast to overcome the undesirable growth inhibition effects of statins. One such mechanism could be active export of statins.

Secondary metabolite gene clusters, in addition to the catalytic enzymes, often encode proteins for secretion of the produced bioactive compounds and thereby also a self-resistance mechanism (reviewed in (Martín et al., 2005)). This is also likely the case for the known statin clusters, where putative efflux pump encoding genes are present; *mlcE* in the mevastatin cluster (Fig. 1A) (Abe et al., 2002b), and *lovI* or *mokI* in the lovastatin cluster of *A. terreus* (Kennedy et al., 1999) or *M. ruber* (Chen et al., 2008), respectively (Fig. 1B). Given the industrial importance of the microbial statin-producing cell factories it is surprising that only limited

evidence concerning the function of the putative efflux pumps in the statin gene clusters has been provided so far. Hutchinson et al. found that *A. terreus lovI* mutants did not produce lovastatin or any of its known precursors, and that heterologous expression of *lovI* in *Aspergillus nidulans*, a lovastatin sensitive species, did not result in increased lovastatin resistance (unpublished result in (Hutchinson et al., 2000)). These findings did not clarify the function of the putative efflux pumps in the statin-producing fungi. Nevertheless, understanding the statin transport mechanism could open up an alternative avenue to classical metabolic engineering strategies aimed at increased productivity of the natural statin-producing strains (Barrios-González and Miranda, 2010). Moreover, genes encoding for the statin transporters can represent a pool of candidates for co-expression in a heterologous host, such as *S. cerevisiae*, thus open up a possibility to establish the necessary self-resistance mechanism for the production of statins in yeast.

In this study, we investigate the function of the putative efflux pump MlcE from the *P. citrinum* mevastatin gene cluster and explore its potential to confer statin resistance in *S. cerevisiae*.

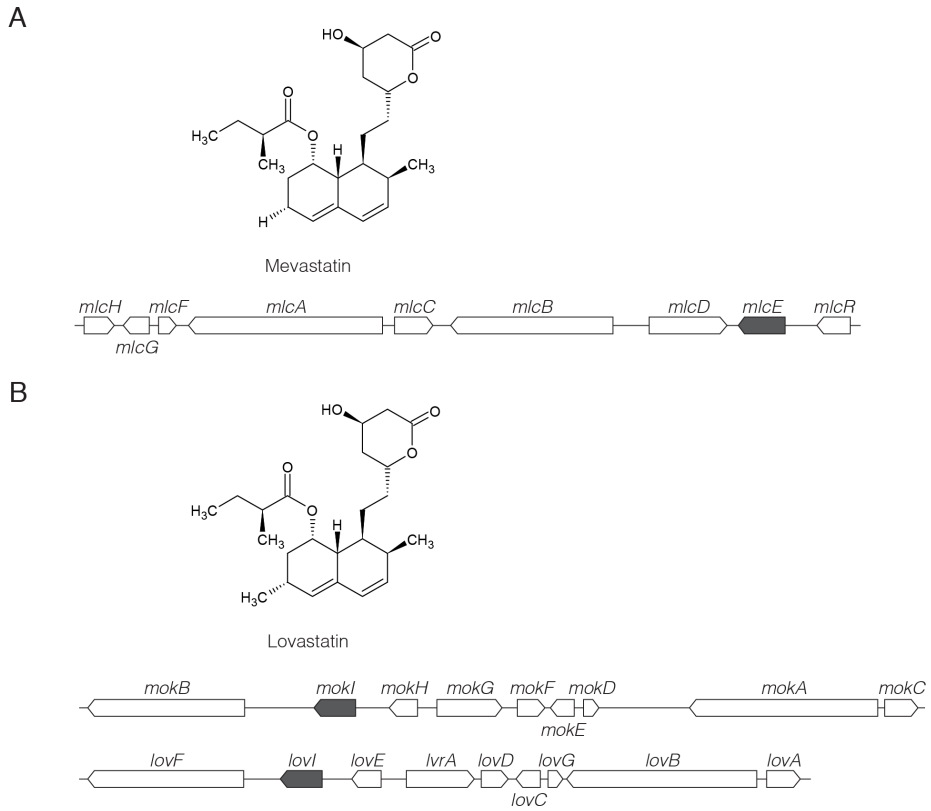

Fig. 1. Natural statins and their biosynthetic gene cluster. (A) Mevastatin and its gene cluster from *P. citrinum.* (B) Lovastatin and its gene clusters from *M. ruber* (*mok* genes) and *A. terreus* (*lov* genes). The putative efflux pump genes are shown in grey.

6.2 Materials and methods

6.2.1 Bioinformatics

Protein sequences were obtained from UniProtKB (Consortium, 2013). Protein topology prediction was carried out using TOPCONS web server (Bernsel et al., 2009). Prediction of subcellular localization was performed with CELLO v.2.5 (Yu et al., 2006). For phylogenetic tree construction the protein sequences were aligned with the multiple sequence alignment tool MAFFT (Multiple sequence Alignment using Fast Fourier Transform) (Katoh et al., 2009) available at the European Bioinformatics Institute (EMBL-EBI) (McWilliam et al., 2013). See Supplementary Table S1 for the list of protein sequences used for the tree construction. The

phylogenetic tree was generated with the ClustalW2 alignment extension (Larkin et al., 2007) at EMBL-EBI using the Neighbor joining clustering method, with the following setting: distance correction on, exclude gaps on. FigTree software, version 1.4 was used for displaying the tree.

6.2.2 Construction of plasmids and strains

A yeast codon-optimized version of the *mlcE* gene, *de novo* synthetized by Genscript, was PCR amplified from the plasmid pEN669 with primers mlcE-F and mlcE-R. The *S. cerevisiae TEF1* promoter was amplified from the plasmid pSP-G2 (Partow et al., 2010) using primers TEF1-d and PGK1-s. The amplified fragments were cloned into the pX-3 targeting vector (Mikkelsen et al., 2012) via the USER cloning technique (Nour-Eldin et al., 2006) resulting in plasmid pX3-TEF1-mlcE-CYC1. The subcellular localization of MlcE was determined by tagging it C-terminally with monomeric red fluorescent protein (RFP). For that plasmid pX3-TEF1-mlcE-RFP-CYC1 and a control plasmid pX3-TEF1-RFP-CYC1 were constructed as follows: the coding sequence of *mlcE* lacking the stop codon was amplified using the primer pair mlcE-F and mlcE-RFP-R, and a yeast codon-optimized *RFP* was amplified from plasmid pWJ1350 (Lisby et al., 2003) using the primers RFP_R+ and either RFP-F (for tagging *mlcE*) or RFP_F+ (for the control plasmid). All fragments were amplified by PCR using a USER cloning compatible PfuX7 polymerase (Nørholm, 2010). *Escherichia coli* DH5α (Woodcock et al., 1989) was used as host for USER cloning experiments and for the propagation of the constructed plasmids. The inserts of the resulting plasmids were verified by sequencing (StarSEQ). The constructed plasmids were digested with the NotI enzyme (New England Biolabs), and the obtained linear fragments were used for yeast transformation using the lithium acetate/single-stranded carrier DNA/polyethylene glycol transformation method (Gietz and Schiestl, 2007). The linear gene targeting cassettes were integrated into the X-3 locus of the reference

yeast strain, *S. cerevisiae* CEN.PK 113-11C as described by Mikkelsen et al (Mikkelsen et al., 2012). The *URA3* markers in the constructed strains were removed by direct repeat recombination using 5-FOA (Melford) counter selection. Correct integration of substrates was verified by diagnostic colony PCR with one primer annealing outside of the integration site in the yeast genome (X-3-up-out-sq), and one substrate specific primer (C1_TADH1_F). Oligonucleotides, plasmids and strains used in this study are listed in Table 1.

Table 1

Oligonucleotides, plasmids and strains used in this study. U = 2-deoxyuridine.

Primer name	**Primer sequence (5`- 3`)**	**Use**
mlcE-F	AGCGATACGUAAAAATGAGTGAACCATTACC	Amplification of *mlcE* from plasmid pEN669
mlcE-R	CACGCGAUTTATGCATCAGTCTCAG	
TEF1-d	ACGTATCGCUGTGAGTCGTATTACGGATCCTTG	Amplification of promoter sequence from plasmid pSP-G2
PGK1-s	CGTGCGAUGCCGCTTGTTTTATATTTGTTG	
RFP_F+	ATGGCCTCCUCCGAGGACGTCATCAAGGAG	Amplification of *RFP* from plasmid pWJ1350
RFP_R+	CACGCGAUCTAGGCGCCGGTGGAGTGGCGG	
mlcE-RFP-R	AGGAGGCCAUTGCATCAGTCTCAGGGAC	Amplification of *mlcE* from plasmid pX3-TEF1-mlcE-CYC1
RFP-F	AGCGATACGUAAAAATGGCCTCCTCCGAG	Amplification of *RFP* from plasmid pX3-TEF1-mlcE-RFP-CYC1
X-3-up-out-sq	TGACGAATCGTTAGGCACAG	Strain confirmation via colony PCR
C1_TADH1_F	CTTGAGTAACTCTTTCCTGTA	
Plasmid name	**Description**	**Reference or source**
pEN669	Template for amplifying *mlcE* (*S. cerevisiae* codon optimized)	from Evolva Holding SA
pWJ1350	Template for amplifying *RFP*	(Lisby et al., 2003)
pSP-G2	Template for amplifying *TEF1*	(Partow et al., 2010)
pX3	USER cloning vector equipped with the *CYC1* terminator designed to target site 3 on chromosome X.	(Mikkelsen et al., 2012)
pX3-TEF1-mlcE-CYC1	Plasmid carrying a gene-targeting cassette for expressing *mlcE* in yeast.	This study
pX3-TEF1-RFP-CYC1	Plasmid carrying a gene-targeting cassette for expressing *RFP*-tagged *mlcE* in yeast.	This study
pX3-TEF1-mlcE-RFP-CYC1	Plasmid carrying a gene-targeting cassette for expressing *RFP* in yeast.	This study
Strain name	**Genotype**	**Reference or source**
Escherichia coli		
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	(Woodcock et al., 1989)
Saccharomyces cerevisiae		
CEN.PK113-11C *(Wild type strain)*	*MATα MAL2-8C SUC2 his3Δ ura3-52*	Dr. Petter Kötter, Institut für Mikrobiologie, der Johan Wolfgang Goethe-Universität, Frankfurt am Main, Germany
ARX1	*MATα MAL2-8C SUC2 his3Δ ura3-52 X3::PTEF1-mlcE-RFP-Tcyc1*	This study
ARX2	*MATα MAL2-8C SUC2 his3Δ ura3-52 X3::PTEF1-RFP-Tcyc1*	This study
ARX3	*MATα MAL2-8C SUC2 his3Δ ura3-52 X3::PTEF1-mlcE-Tcyc1*	This study

6.2.3 Media

The *E. coli* transformants were selected on lysogeny broth (LB) medium containing 100 µg/mL of ampicillin. Yeast strains were cultivated in standard liquid or solid yeast peptone dextrose medium (YPD), synthetic complete medium (SC), or synthetic medium (SM). SC medium was prepared according to Sherman et al. (Sherman et al., 1986), with the minor modification that the L-leucine concentration was doubled to 60 mg/L. Yeast transformants were selected on SC medium lacking uracil. Removal of the *URA3* marker, via direct repeat recombination, was achieved by growing the strain on SC medium containing 5-fluororotic acid (5-FOA; 740 mg/L, Sigma-Aldrich) and uracil (30 mg/L).

For susceptibility experiments strains were grown aerobically either on YPD plates or in SM, supplemented with compounds as described below. SM was prepared according to Verduyn (Verduyn et al., 1992), but concentrations of $(NH_4)_2SO_4$ and KH_2PO_4 were modified to 7.5 g/L and 14.4 g/L, respectively. SM was supplemented with uracil (150 mg/L; Sigma-Aldrich) and L-Histidine (125 mg/L; Sigma-Aldrich) (Pronk, 2002). The pH was adjusted to 6.5 with a 2M NaOH solution. Glucose was added as carbon source to a final concentration of 20 g/L. The compounds used in the susceptibility experiments were prepared as follows: stock solutions of vanillin (320 mM), mycophenolic acid (MPA, 50 mM) and atorvastatin (10 mM) were prepared by dissolving the compounds in 99% ethanol. Mevastatin, lovastatin, and simvastatin stock solutions (50 mM) were prepared as described previously (Morimoto et al., 2013). Briefly, the solid compounds were dissolved in 1 mL of 99% ethanol, preheated to 50°C, alkalinized with 0.5 mL of 0.6 M NaOH and incubated at 50°C for 2 hours. The pH of the solutions was then adjusted to 7.2 by adding 0.4 M HCl. The final volume of the solutions was adjusted to 2 mL with water, resulting in stock solutions of 50 mM. All stock solutions were filter-sterilized and stored at -20°C. Mevastatin and atorvastatin were purchased from Toronto

Research Chemicals, lovastatin from Tokyo Chemical Industry, MPA and vanillin from Sigma-Aldrich, and simvastatin from Ark Pharm.

6.2.4 Fluorescent Microscopy

For fluorescent microscopy the *mlcE-RFP*- and *RFP*-expressing strains (ARX1 and ARX2, respectively) were cultured in liquid SC medium at 30°C with 150 rpm agitation overnight and analyzed by fluorescence and visible light microscopy using a Nikon Eclipse E1000 microscope equipped with an oil-immersed objective at 100 x magnification. The images were captured with QImaging Retiga Exi digital camera using Image Pro Plus 5.1 software. The brightness of images to be compared was adjusted pairwise using Adobe Photoshop CS6.

6.2.5 Susceptibility experiments

For susceptibility assays on solid media tenfold dilution series of *S. cerevisiae* WT and ARX3 strains (Table 1), starting with an OD_{600} of 0.02 were prepared from overnight cultures in SC medium (30°C/150 rpm). 4.5 µL of each dilution were plated on a set of YPD agar plates containing different cytotoxic compounds. The plates were incubated at 30°C for 3 days, after which the growth of the yeast strains was recorded by photography.

For susceptibility assay in liquid medium, strains were grown aerobically in SM, containing different concentrations of lovastatin. Yeast optical density measurements were performed in 48 wells plates in a plate reader (BioTek's Synergy™ Mx Microplate Reader) at 30°C with fast shaking intensity setting (19 Hz speed, linear shake, which translates into 1140 rpm according to the BioTek`s instructions) in 400 µL of SM. Cells were harvested from overnight shake flask cultures (30°C/150 rpm) in late exponential phase and diluted to an OD_{600} of 0.1 in SM medium with 0.7, 1.2 or 2.0 mM of activated lovastatin or an equal volume of

control solution (99% ethanol treated as described above – preparation of the compounds for the susceptibility experiments). Triplicate OD_{600} measurements were taken every 5 minutes for 24 hours.

6.3 Results and Discussion

6.3.1 Topology prediction and phylogenetic clustering of MlcE

MlcE, a putative efflux pump from the *P. citrinum* mevastatin biosynthetic gene cluster shows significant sequence similarity to drug resistance proteins of the major facilitator superfamily (MFS) (Marger and Saier, 1993). MFS transporters are characterized by using the proton gradient across the plasma membrane as an energy source for the translocation they mediate (Pao et al., 1998). The drug resistance protein subfamily of MFS transporters is further divided into two families, depending on the number of transmembrane spanning regions (TMS) the proteins consist of; 12-TMS family and 14-TMS family (Paulsen and Skurray, 1993; Paulsen et al., 1996), also termed as Drug:H^+ antiporter 12 TMS (DHA12) family, and 14 TMS (DHA14) family, respectively (Pao et al., 1998). The performed phylogenetic analysis showed that MlcE, as well as LovI and MokI (Fig. 2), clustered with known members of 14-TMS family of drug resistance proteins, such as the cercosporin facilitator protein (CFP) from *Cercospora kikuchii* (Callahan et al., 1999) and HC-toxin efflux pump (ToxA) from *Cochliobolus carbonum* (Pitkin et al., 1996) (further proteins are listed in Supplementary Table S1). This classification is supported by the performed topology prediction, which showed that MlcE comprises of 14 TMS (data not shown), indicating that it is indeed a member of 14-TMS family. We next performed an *in silico* prediction of MlcE's subcellular localization, using CELLO v.2.5 to see where in eukaryotic cells the protein would be localized. The prediction suggests that it is most likely localized at the plasma membrane (score = 4.942 and a

combined reliability score of 0.997 for the five used prediction methods). Collectively, this proposes that MlcE is likely localized in the plasma membrane and functions as a statin efflux pump driven by the proton gradient found across the plasma membrane.

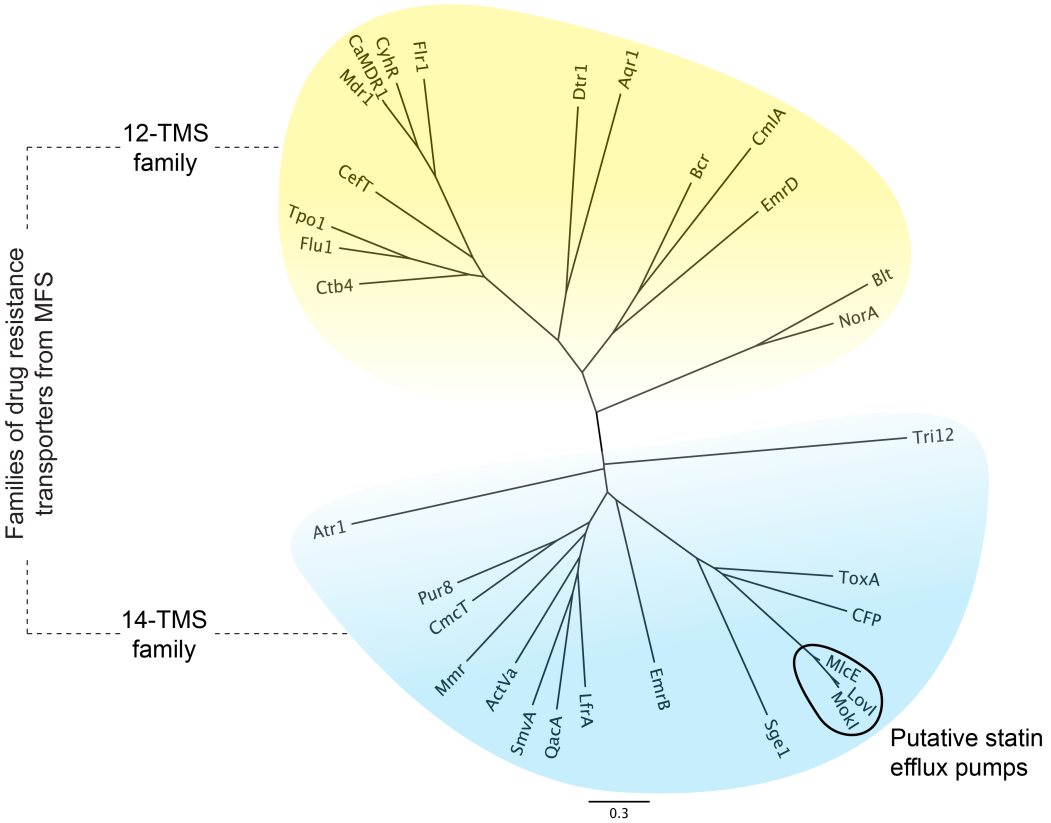

Fig. 2. Phylogenetic clustering of putative statin efflux pumps (MlcE, MokI and LovI) with major facilitator superfamily (MFS) transporters involved in the efflux of toxic compounds, belonging to the subfamily of proteins with 14 transmembrane domains (14-TMS family). Proteins used to construct the phylogenetic tree are listed in Supplementary Table S1, where information about the source organism and the substrate of each protein is provided.

6.3.2 Subcellular localization of MlcE

To experimentally determine the subcellular localization of MlcE in *S. cerevisiae* we tagged MlcE with the red fluorescent protein (RFP) at its carboxylic terminus and expressed it as a single copy gene from the yeast genome (Fig. 3A). Fluorescent microscopy of the resulting strain, ARX1, revealed a ring-like

distribution of the fluorescent protein at the periphery of the cells and inside the vacuole (Fig. 3B), indicating that the RFP-tagged MlcE was localized to the plasma and vacuolar membranes. In contrast, when RFP was expressed alone it was found to have a uniform cytoplasmic distribution in the control cells ARX2. This subcellular localization of MlcE in *S. cerevisiae* supports the hypothesis that MlcE is a transmembrane protein, which is localized to the plasma membrane.

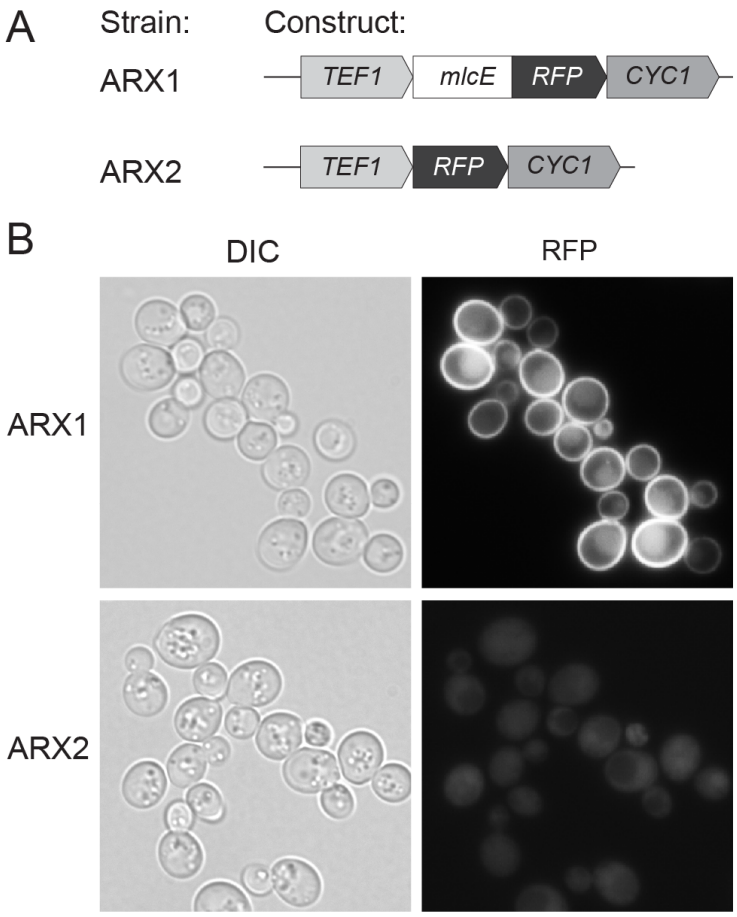

Fig. 3. Subcellular localization of MlcE in *S. cerevisiae.* (A) Strain construction summary. (B) Fluorescent microscopy of the constructed strains (see Materials and methods for experimental details).

6.3.3 Investigation of the potential of MlcE to confer the resistance to statins in *S. cerevisiae*

We next tested if the localization of MlcE to the yeast plasma membrane would enable it to export statins from yeast, and thereby increase the yeast`s resistance to statins. For that, *mlcE* was expressed from a defined genomic locus in *S. cerevisiae* as a single copy gene under the control of the strong constitutive promoter TEF1 (Fig. 4A). The resulting strain ARX3 was tested for its susceptibility to mevastatin, MlcE's predicted natural substrate, by serial dilution plating on YPD agar plates supplemented with the active form of mevastatin. The *mlcE*-expressing strain showed an increased resistance to mevastatin compared to the reference strain (Fig. 4B).

To determine if MlcE would be able to excrete other structurally related compounds, we tested the effects of lovastatin and simvastatin. Again, the *mlcE*-expressing strain displays an increased resistance compared to the reference strain, and the putative pump was able to protect the cells against both the natural statin lovastatin and its semi-synthetic derivative simvastatin (Fig. 4B). This shows that MlcE is able to accept not only its native substrate but also structurally related natural compounds, and even compounds it has not encountered during evolution, when expressed in yeast. To determine whether MlcE should be considered as a general pleiotropic efflux pump, or a dedicated statin pump, we tested the susceptibility of the ARX3 strain to other toxic compounds. This analysis showed that MlcE was not able to protect yeast against the lethal effects of the synthetic statin, atorvastatin or the effects of the natural compounds vanillin and mycophenolic acid (MPA) (Fig. 4B). These results suggests that MlcE is not a multi-drug resistance efflux pump. The specificity of MlcE and its presence in the mevastatin biosynthetic gene cluster suggest that it has likely evolved as a statin efflux pump; however, testing in the endogenous species is still required to confirm this.

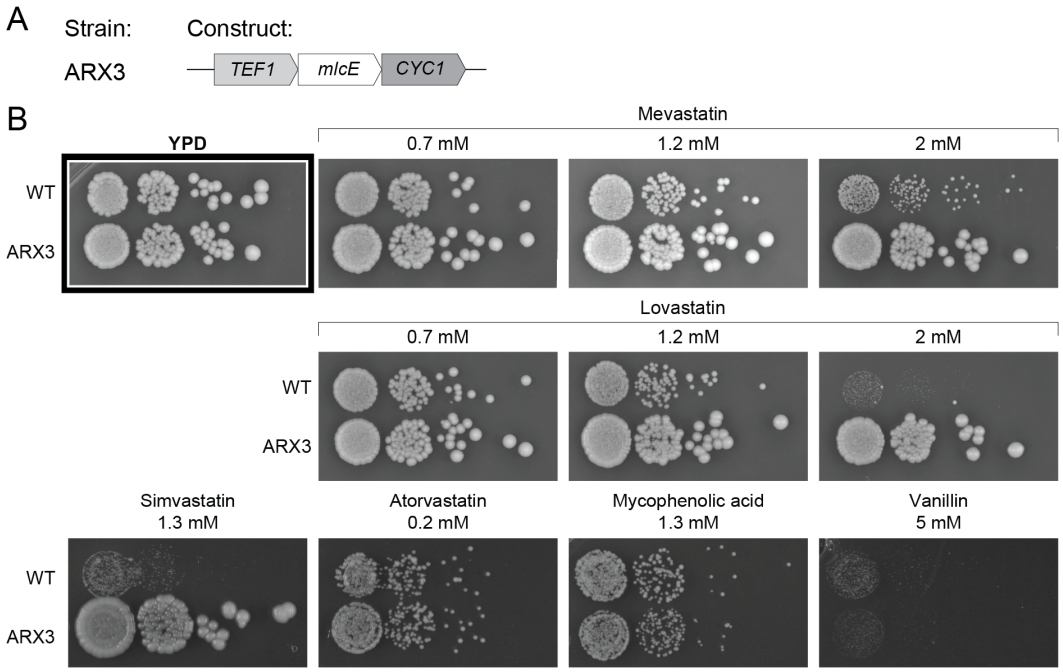

Fig. 4. Investigation of the potential of MlcE to confer the resistance to statins in *S. cerevisiae.* (A) Strain construction summary. (B) Susceptibility assay. Ten-fold dilution series of WT (CEN.PK 113-11C) and ARX3 strains (harboring MlcE efflux pump), starting with and OD_{600} of 0.02 were prepared from overnight cultures and plated on a set of YPD agar plates containing different cytotoxic compounds. The plates were incubated at 30°C for 3 days, after which the growth of the strains was recorded by photography. The plate in the black square represents the reference plate (no compounds added to YPD) (for experimental details see Materials and methods).

6.3.4 MlcE and a future *S. cerevisiae* based statin cell factory

Statins are currently commercially produced by fermentation of natural statin-producing species of filamentous fungi. The highest titers reported for these systems have been achieved by submerged cultivation, reaching levels up to 950 mg/L (2.35 mM) of lovastatin (Jia et al., 2010) and 1200 mg/L (3.07 mM) of mevastatin (Choi et al., 2004), respectively. For future heterologous production of statins to be competitive, these titers will likely have to be matched and preferably exceeded. For the last decade several groups have been working on establishing *S. cerevisiae* based statin cell factories, and in 2013 Xu et al. succeed in producing dihydromonacolin L acid (0.11 mM), the first stable intermediate in the lovastatin

pathway (Xu et al., 2013). However, no one has yet reported whether *S. cerevisiae* will be able to cope with the required product levels. To test this, we cultured the reference wild type strain (WT) in liquid synthetic medium supplemented with increasing concentrations of activated lovastatin in a micro-fermentation setup. The analysis revealed that the IC_{50} value for extracellularly added lovastatin is approx. 1 mM (less than half of the required concentration) in the wild type and that even low concentrations of lovastatin greatly reduced the aerobic maximum specific growth rate and final optical density (Table 2).

These results show the necessity of establishing a non-destructive self-resistance mechanism in a future yeast statin cell factory to allow for titers similar to those reported for statin-producing filamentous fungi. For this, MlcE constitutes a potential tool for tackling the described self-intoxication problem. To investigate if it would also provide protection from statins in liquid cultures, the MlcE expressing strain (ARX3) was tested as described above for the reference wild type strain (Table 2). The analysis showed that while the growth of *S. cerevisiae* wild type strain was almost completely inhibited at lovastatin concentrations similar to those achieved by fermentation of *A. terreus*, growth of the ARX3 strain was only slightly affected by the same high concentration of lovastatin. The liquid culture experiment also allowed for determination of the strains growth efficiencies (Table 2), which revealed that expression of *mlcE* did have a cost (9% reduction), but that this cost did not change as function of the statin concentration, within the tested concentration range.

Direct proof of the effects of implementing the MlcE based resistance in a *S. cerevisiae* statin cell factory is currently not possible as only part of the biosynthetic pathway at this time has been established in yeast (Xu et al., 2013). However, implementation could likely have additional benefits such as increasing titers by reducing feedback inhibition of the pathway enzymes caused by high intracellular concentrations of statins and furthermore reduce product purification costs.

Table 2

Aerobic maximum specific growth rates and growth efficiencies calculated as $\Delta(OD_{600,max}\text{-}OD_{600,t=0})$ of *S. cerevisiae strains* WT (CEN.PK 113-11C) and ARX3 (harboring MlcE efflux pump) on glucose and different concentrations of activated lovastatin. In the samples with 0 mM lovastatin, an equal volume of solvent was added. Averages and standard deviations were obtained from triplicate experiments.

Lovastatin concentration (mM)	Growth rate (h^{-1})		Growth efficiency	
	WT	ARX3	WT	ARX3
0	0.28 ± 0.004	0.31 ± 0.01	0.90 ± 0.008	0.82 ± 0.04
0.7	0.18 ± 0.01	0.34 ± 0.008	0.37 ± 0.04	0.87 ± 0.02
1.2	0.10 ± 0.008	0.27 ± 0.003	0.28 ± 0.01	0.84 ± 0.02
2.0	0.04 ± 0.005	0.27 ± 0.005	0.10 ± 0.01	0.86 ± 0.02

6.4 Conclusions

We provide evidence that *mlcE* from the *P. citrinum* mevastatin biosynthetic gene cluster encodes a transmembrane protein that localizes to the plasma and vacuolar membranes in *S. cerevisiae*. Moreover, MlcE significantly increases yeast resistance to both, natural and semi-synthetic statins, likely by exporting the compounds from the cells. This resistance mechanism has a potential to improve future yeast based production of natural and semi-synthetic statins.

Acknowledgements

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6.5 References for Chapter 6

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Heterologous expression of MlcE in *Saccharomyces cerevisiae* provides resistance to natural and semi-synthetic statins

Supplementary information (to Chapter 6)

Ana Ley[a], Hilde Cornelijne Coumou[a], Rasmus John Normand Frandsen [a§]

[a]Department of Systems Biology**,** Technical University of Denmark, Søltofts Plads 223, 2800 Kgs. Lyngby, Denmark

[§]Corresponding author: Rasmus John Normand Frandsen, E-mail: rasf@bio.dtu.dk, Telehone: 0045 45252708, FAX: 0045 45884148.

Email addresses of non-corresponding authors:

Ana Ley: anare@bio.dtu.dk

Hilde Cornelijne Coumou: hilco@bio.dtu.dk

Table S1

Drug resistance proteins used for the construction of the phylogenetic tree. Proteins are divided into two families of the major facilitator superfamily (MFS) transporters: Drug:H^+ antiporter family 1 (DHA12) with 12 transmembrane-spanning regions (12TMS) and Drug:H^+ antiporter family 2 (DHA14) with 14 transmembrane-spanning regions (14TMS).

Protein name	Microorganism	Representative Substrate	Accession Number (UniProtKB)
MFS - DHA14 (14 TMS)			
ToxA	*Cochliobolus carbonum*	HC-toxin	Q00357
Tri12	*Fusarium sporotrichioides*	Trichotechene	O93842
CFP	*Cercospora kikuchii*	Cercosporin	O93886
Atr1	*Saccharomyces cerevisiae*	Aminotriazole	P13090
Sge1	*Saccharomyces cerevisiae*	Crystal violet	P33335
EmrB	*Escherichia coli*	Carbonyl cyanide m-chlorophenyl hydrazone	P0AEJ0
LfrA	*Mycobacterium smegmatis*	Acriflavin	Q50392
QacA	*Staphylococcus aureus*	Benzalkonium chloride	Q1XG09
SmvA	*Salmonella typhimurium*	Ethidium bromide	P37594
ActVa 1	*Streptomyces coelicolor*	Actinochodrin	Q53903
CmcT	*Nocardia lactamdurans*	Cephamycin	Q04733
Mmr	*Streptomyces coelicolor*	Methylenomycin A	P11545
Pur8	*Streptomyces lipmanii*	Puromycin	P42670
MFS - DHA12 (12 TMS)			
Ctb4	*Cercospora nicotinae*	Cercosporin	A0ST42
CefT	*Acremonium chrysogenum*	Cephalosporin	Q8NKG7
Mdr1	*Canidida albicans*	Fluconazole	P28873
Flu1	*Candida albicans*	Fluconazole	G1UB37
Bcr	*Escherichia coli*	Bicyclomycin	C6EA15
Blt	*Bacillus subtilis*	Acriflavin	M1U4Q0
EmrD	*Escherichia coli*	Carbonyl cyanide m-chlorophenyl hydrazone	P31442
CaMDR1	*Candida albicans*	Benomyl	Q9URI2
NorA	*Staphylococcus aureus*	Acriflavin	P0A0J7
CyhR	*Candida maltosa*	Cycloheximine	P32071
CmlA	*Pseudomonas aeruginosa*	Chloramphenicol	Q83V15
Flr1	*Saccharomyces cerevisiae*	Fluconazole	P38124
Tpo1	*Saccharomyces cerevisiae*	Spermine	Q07824
Dtr1	*Saccharomyces cerevisiae*	Dityrosine	P38125
Aqr1	*Saccharomyces cerevisiae*	Quinidine	P53943
Putative Statin Efflux Pumps - unknown family			
MlcE	*Penicillium citrinum*	Mevastatin	Q8J0F3
LovI	*Aspergillus terreus*	Lovastatin	Q9Y7D4
MokI	*Monascus pilosus*	Monacolin	Q3S2U5

Overall Conclusions and Perspectives

One of the future perspectives in biotechnological production of natural and semi-synthetic statins is in the use of a fast-growing heterologous host, such as *Saccharomyces cerevisiae*. The focus of my research was on the development of *S. cerevisiae* cell factory for the production of compactin, a natural statin that is synthesized as a secondary metabolite by the filamentous fungus *Penicillium citrinum*. I covered two different aspects of the cell factory development; heterologous expression of the compactin biosynthetic pathway in *S. cerevisiae* and establishing a statin resistance mechanism in *S. cerevisiae*.

In Chapter 4 I describe my work aimed at the production of compactin in *S. cerevisiae*. I used a chromosomal gene integration approach to express the compactin biosynthetic genes (*mlcA, mlcC, mlcF* and *mlcG*), which are required for the synthesis of the compactin nonaketide intermediate ML-236A, as well as two putative self-resistance genes (*mlcE* and *mlcD*). Two different PPTases (i.e. *npgA* and *sfp*), required for the posttranslational modification of the PKS MlcA were co-expressed individually in the strain harboring the four compactin biosynthetic genes. However, the constructed strains did not synthesize the expected intermediates. Fluorescence microscopy of strains expressing RFP-tagged MlcA suggested that MlcA accumulated as protein aggregates. The latter can arise from improper folding of the protein, a situation that could explain the inability of MlcA to catalyze the polyketide formation. Lowering the cultivation temperature, an action that can positively affect protein folding, did not result in the production of compactin intermediates. Additional studies of the expression of the required proteins, their localization, and post-translational modification need to be carried out to understand the problem and find a suitable solution. My ambition to develop a yeast

cell factory for compactin production was not accomplished, however Xu *et al.* succeeded in production of dihydromonacolin L acid, first stable lovastatin intermediate, in *S. cerevisiae*, suggesting that the future production of statins in yeast is possible. With the broad availability of molecular genetic manipulation tools, as well as synthetic biology and metabolic engineering tools, production of statins in yeast will open an avenue of opportunities for optimization of statin production. Moreover, advances in the understanding the polyketide biosynthetic mechanisms has in other systems enabled manipulation of both, the synthesis of the polyketide backbones by PKSs and the post-PKS synthesis modifications by tailoring enzymes, which resulted in production of entirely new compounds. Together with the combinatorial biochemistry approach, such manipulations could be also applied for the biosynthesis of new statins with alter pharmacokinetic and pharmacodynamics properties. On this matter, the synthesis of statins in *S. cerevisiae* would provide a much better platform than natural producers, filamentous fungi, due to the ease of genetic manipulation and absence of native secondary metabolism, which can complicate polyketide biosynthesis due to cross-reactivity.

Construction of a *S. cerevisiae* cell factory for the production of high concentrations of statins will require the establishment of a non-destructive self-resistance mechanism to overcome the undesirable growth inhibition effects of statins. The second aspect of my research activities focused on finding solutions for tackling the problem with *S. cerevisiae* statin-sensitivity. In Chapter 5 I investigated the potential of the putative HMGCR MlcD, from the *P. citrinum* biosynthetic gene cluster, to confer the statin resistance in *S. cerevisiae* and I aimed at elucidating the molecular biological basis behind *mlcD*-derived statin resistance. I showed that *mlcD* can mediate statin resistance when expressed heterologously in *S. cerevisiae*. Successful complementation of *Sc-HMG1* and *Sc-HMG2* in yeast, in addition, provides evidence that MlcD functions as HMGCR. Next I investigated the mechanism of the self-resistance provided by MlcD; do natural statin producers avoid self-intoxication by increasing the concentration of HMGCR in the cell at the

time of statin synthesis, and thus reducing the relative concentration ratio between the bioactive compound and the target protein? Or do the statin producers possess a unique version of HMGCR, possibly statin-insensitive? I addressed this question with the use of bioinformatics. The phylogenetic clustering of fungal HMGCRs and analysis of their domain organization suggested that the three HMGCRs from the known statin gene clusters (*mlcD and lvrA/mokG*) are likely derived from HMGCRs involved in primary metabolism. However, the occurrence of these genes in the different statin gene clusters probably did not arise from a recent duplication of the primary HMGCR. Instead, I propose that the HMGCR-encoding genes must at some time during evolution have duplicated and then recruited to the statin gene clusters, a situation that has increased the chance for becoming co-regulated with the cluster and hence statin production. Results obtained with the bioinformatics approach suggest that the statin self-resistance mechanism is based on the association with the cluster, e.g. increased concentration of HMGCR at the right time, and not on the expression of the statin-insensitive version of HMGCR. However, this question remains unresolved and further biochemical characterization of the HMGCRs from the statin-clusters is required to determine their statin sensitivity. Comparison of the statin binding affinities (Ki values) for the HMGCRSs from the statin clusters and for the ones encoded elsewhere in the genome will provide insight into the statin resistance mechanism.

Work described in Chapter 6 also concerns the resistance mechanism, but it includes expression of another putative resistance gene from the compactin cluster, namely *mlcE*, in *S. cerevisiae*. I provide evidence *mlcE* encodes a transmembrane protein that localizes to the plasma and vacuolar membranes in *S. cerevisiae*, and most importantly, MlcE significantly increases yeast resistance to both, natural and semi-synthetic statins, likely by exporting the compounds out of the cells.

The work presented in Chapter 5 and 6 shows that understanding the molecular biological basis underlying the self-resistance mechanisms in secondary metabolite producers can provide tools for improvement of biotechnological

processes. Solutions for tackling the problem with *S. cerevisiae* statin sensitivity could have an enormous impact on the future production of statins in yeast. I show that both MlcD and MlcE can improve the yeast statin resistance, and thus provide means for improvement of this cell factory for future statin production. Besides the improved statin resistance, expression of *mlcE* in *S. cerevisiae* has a potential for reducing purification cost, and also removing the negative feedback inhibition that sometimes limits the biosynthesis of the end product.

Fungal secondary metabolite gene clusters are not just an important source of enzymes for the synthesis of valuable compounds, but can also contain genes for the self-resistance mechanisms, which are largely unexplored. Like in the case of statin gene clusters, other fungal gene clusters could also represent a good source for identifying the resistance genes and discovering the underlying resistance mechanisms that can protect heterologous cell factories from the toxic compounds, and thus ensure an efficient production of desired secondary metabolites.

APPENDIX: Patent

STATIN RESISTANCE AND EXPORT

Technical field of the invention

The present invention relates e.g. to methods of producing statins in transgenic, non-filamentous microorganisms such as *Saccharomyces cerevisiae*. Further, the present invention relates to the transgenic, non-filamentous microorganisms *as such* as well as various uses of transmembrane statin efflux pump(s) originating from various filamentous fungi.

Background of the invention

Statins are important inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), the regulatory and rate-limiting enzyme in the mevalonate pathway, which leads to the production of sterols, such as cholesterol in human, and ergosterol in fungi.

The blood cholesterol level in mammals is a result of *de novo* synthesis and dietary intake. Elevated levels of blood cholesterol often lead to atherosclerosis, i.e. deposits of LDL particles on the inside of the arterial walls, leading to various cardiovascular diseases. Treatment of elevated cholesterol levels is typically a combination of dietary changes and medical treatment with statins to control *de novo* synthesis.

With their effective cholesterol-lowering ability, statins have been widely used as hypercholesterolemia drugs to prevent and treat cardiovascular diseases and has become one of the best-selling pharmaceuticals in the past decade.

Typically, statins are divided into three classes based on their mode of synthesis: natural, semi-natural and synthetic. Natural statins, such as lovastatin/monacolin K and compactin, are synthesised via the polyketide biosynthetic pathway by filamentous fungi including *Aspergillus terreus, Monascus purpureus* and *Penicillium citrinum*. The natural statin compounds are utilized by the fungi to inhibit the growth of eukaryotic competitors that inhabits the same ecosystems/niche. Semi-natural statins are natural statins, which post-purification

has been modified through synthetic chemistry or via a biotransformation. The synthetic statins differ significantly in structure from that of the natural and semi-natural statins and are produced by chemical synthesis.

More specifically, the two known natural statins, compactin and lovastatin, are produced as secondary metabolites by filamentous fungi; compactin is produced by *Penicillium* species, e.g. *P*. *solitum* and *P. brevicompactum,* whereas lovastatin is produced by *Aspergillus* and *Monascus species*, e.g. *A. terreus*, *M. purpureus* and *M. pilosus.*

In general, bioactive secondary metabolites often provide a selective advantage to the producing microorganism in their natural environment. However, said metabolites can also be toxic to the producing microorganisms if they themselves contain the target site of the compound. Therefore, the secondary metabolite biosynthesis gene clusters, in addition to the biosynthetic enzymes, often also contain genes encoding a secretion system(s), which in addition can provide a resistance mechanism to prevent self-intoxication.

This is also the case in relation to both the compactin and the lovastatin biosynthesis gene clusters, where putative efflux pump genes have been identified, namely the *mlcE* gene from the compactin biosynthetic gene cluster, *lovI* gene (also referred to in the art as: ORF10 or *lovH* - thus, for the purpose of simplicity the gene is hereinafter referred to as *lovI/H*) from the lovastatin gene cluster and *mokI* from the monacolin K cluster.

Well-known methods of producing natural statins, as well as their semi-synthetic derivatives, are mainly based on fermentation processes with strains of naturally statin-producing filamentous fungi.

Commercial production of natural and semi-natural statins is based on liquid fermentation of the relevant fungal species followed by purification and subsequent modification for the semi-natural statins. However, it is also well known that filamentous fungi are difficult to culture efficiently in fermenters, *inter alia* due to their unique physiology and morphology.

Hence, in order to overcome these problems and to increase the yields, several statin-manufacturing companies have switched to solid state fermentation, a challenging approach that is prone to contamination and involves a relatively high risk for the formation of undesirable side products. This is also well known in the art.

Also, it is well-known in the art that there is a common problem while fermenting statins in fungi as the final products are - besides being cholesterol lowering agents - also active antifungals and thereby limit the productivity in fungal hosts. A possible solution to this problem could be to transfer the metabolic pathway to easily fermentable unicellular microorganisms, such as yeast. However, this solution is not easily achievable *inter alia* since yeast does not naturally produce any polyketides, which is one of the reasons why the relevant genes encoding the biosynthetic machinery for the formation of statins have to be functionally expressed simultaneously at a balanced level. Additional challenges for producing statins in yeast include a limited availably of the necessary substrates (acetyl-CoA and malonyl-CoA) and co-factors (NADPH). Further challenges includes problem with self-intoxication as yeast only has a basal level of statin-resistance (Riccardo & Kielland-Brandt, 2011).

Thus, in an effort to provide an alternative mode of biosynthesis, researcher has for the last decade been working on transferring the statin biosynthetic pathway from the traditionally used filamentous fungi into easily fermentable microorganisms such as *Saccharomyces cerevisiae.*

Xu W. et al. (2013), discloses *inter alia* the expression in yeast of the genes responsible for the biosynthesis of the lovastatin intermediate, monacolin J acid. However, the Xu W. et al. -article did not contain any disclosure of the expression of the statin efflux pump genes *mlcE, mokI* and/or *lovI/H* in e.g. *Saccharomyces cerevisiae*, but merely discloses the expression of genes responsible for the biosynthesis of monacolin J acid which is an intermediate capable of being converted into the commercially attractive agent, simvastatin acid, in a single enzymatic step.

Abe et al. (2002) discloses *inter alia* that *mlcE* is a putative efflux pump which may be involved in conferring resistance to compactin as well as in metabolite secretion in the naturally producing microorganism (*Penicillium citrinum*). However, the Abe et al. -article contains no disclosure or suggestions of transferring and/or expressing the *mlcE, mokI* or *lovI/H* genes in yeast, let alone in *Saccharomyces cerevisiae.*

An article by Hirata D., & Yano K. (1994), discloses *inter alia* that the *pdr5* gene encodes an efflux pump in *Saccharomyces cerevisiae*.

Likewise, in an article by Riccardo L., & Kielland-Brandt MC. (2011) is disclosed that *pdr5* gene encodes a pump that has shown to confer basic-level of statin-resistance in *Saccharomyces cerevisiae*. Also disclosed in said article is the susceptibility to lovastatin of *Saccharomyces cerevisiae* strains deleted for *PDR* genes, i.e. genes encoding for drug resistance pumps responsible for exporting hydrophobic and amphiphilic drugs, such as lovastatin.

WO09133089 A1 disclosed *inter alia* a process for increasing the compactin, pravastatin, lovastatin and/or simvastatin productivity by a fermentation process carried out with host organisms that are genetically engineered to have increased resistance to said statins. More specifically, a process is provided which makes use of microorganisms (preferably *Penicillium chrysogenum*) in which genes encoding proteins which mediate statin resistance are overexpressed. Also disclosed in WO09133089 A1 is the compactin biosynthetic gene cluster of *Penicillium citrinum* (i.e. *mlcA, mlcB, mlcC, mlcD, mIcE, mlcF, mlcH, mlcG, mlcR*) as well as the lovastatin biosynthetic gene cluster of *Aspergillus terreus* (i.e. *ORF1, ORF2, lovA, ORF5, lovC, lovD, ORF8, lovE, ORF10, lovF, ORF12, ORF13, ORF14, ORF15, ORF16, ORF18*). However, WO09133089 A1 contains no disclosure of neither the functions of said genes nor the transferring and expression of the statin efflux pump encoding *lovI/H, mokI* or *mlcE* genes in *Saccharomyces cerevisiae*.

WO0129073A1 disclosed to the use of so-called MFS-transporters (named PUMPI and PUMP2) and their ability to confer resistance to otherwise toxic levels of lovastatin when expressed in *Saccharomyces cerevisiae*. However, as there is no disclosure or suggestion in WO0129073A1 of transferring and expressing the

statin efflux pump encoding *lovI/H, mokI* or *mlcE* genes in *Saccharomyces cerevisiae*.

WO0037629 disclosed *inter alia* a method of increasing the production of lovastatin in a lovastatin-producing or a non-lovastatin-producing microorganism. ORF10 (*lovI/H*) is disclosed in WO0037629 as a gene relevant for the transportation of metabolites. There is no disclosure in WO0037629 of *lovI/H, mokI* or *mlcE*, let alone of the transfer and expression of said genes in a *Saccharomyces cerevisiae* host e.g. for improving the statin resistance in said host.

WO2009/077523 disclosed *inter alia* a method for the fermentative production of e.g. compactin (mevastatin) and lovastatin where said method comprises culturing a mutant host capable of producing e.g. lovastatin wherein the esterase activity in said mutant host is more than 25% below the activity of said esterase in the parent host. However, there is no disclosure in WO2009/077523 of e.g. the *mlcE, mokI* and/or *lovI/H* genes encoding statin specific efflux pumps let alone of transferring and expression of said genes in *Saccharomyces cerevisiae*.

WO2007147827 discloses *inter alia Saccharomyces cerevisiae* containing a compactin biosynthesis gene and a gene for conversion of compactin into pravastatin. The *mlcE* gene is disclosed as being one of these compactin biosynthesis genes. There is no disclosure or suggestion in WO2007147827 that e.g. *mlcE, mokI* and/or *lovI/H* is capable of encoding statin specific efflux pumps *in Saccharomyces cerevisiae*, let alone of the transferring and expression of said genes in *Saccharomyces cerevisiae.*

WO2010034686 discloses *inter alia* a method for the fermentative production of e.g. compactin (mevastatin) and lovastatin where the method involves culturing a host, e.g. *Saccharomyces cerevisiae*, e.g. by use of the *lovE* transcription regulator gene. There is, however, no disclosure or suggestion in WO2010034686 of e.g. the *mlcE, mokI* and/or *lovI/H* genes is/are capable of encoding statin specific efflux pumps in e.g. *Saccharomyces cerevisiae*, let alone of a transfer and expression of said genes in *Saccharomyces cerevisiae.*

WO10069914 discloses a method for the fermentative production of e.g. compactin (mevastatin) and lovastatin where the method involves culturing a host, e.g. *Saccharomyces cerevisiae*, e.g. by use of specifically defined transcription regulator genes.

WO10069914 also discloses that *mlcE* encoding an efflux pump in *Penicillinum citrinum*. However, WO10069914 contains no disclosure or suggestions that said efflux pump gene can be expressed in e.g. *Saccharomyces cerevisiae*.

As is apparent from the above-outlined prior art documents, the three genes *mlcE, mokI* and *lovI/H* have not previously been characterized in depth, let alone when transferred into other host organisms. The article by Hutchinson et al. (2000), discloses the function of the *lovI* gene and states *inter alia* that heterologous expression of the putative lovastatin efflux pump gene *lovI* in *Aspergillus nidulans* did not result in increased resistance to lovastatin in said host organism (no experimental data is provided in the article). Moreover, said article contains no disclosure of e.g. expressing the *mlcE, mokI* and/or *lovI*/H genes in yeast.

It is well known that statins are toxic for the statin-producing host cells, e.g. due to the inhibition of ergosterol biosynthesis (fungal equivalent of cholesterol). It is therefore crucial to establish a nondestructive resistance mechanism in a given host cell (said host cell is also commonly referred to as a "cell factory") in order to establish a commercially profitable production of statins.

In order to avoid the undesirable effects of self-intoxication in the host cell several approaches has previously been utilized the most common being: 1) overexpression of the HMGR encoding gene and/or 2) development of a statin-insensitive HMGR.

The present invention relates to a novel approach for avoiding the undesirable effects of self-intoxication in easily fermentable host microorganisms by introduction of a transmembrane statin efflux pump in said microorganism for removing the toxic statins from said host.

This novel approach has the additional advantage that it also ensures the export out of microorganism of any produced statins, which *inter alia* eases the subsequent purification steps.

Hitherto, however, it has not been clear whether the putative efflux pumps from the statin biosynthetic gene clusters have the potential to export statins out of the statin-producing microorganisms. Thus, the inventors of the present application surprisingly found that introduction of genes encoding transmembrane statin efflux pumps, such as the *mlcE, mokI* and/or *lovI/H* gene(s) into statin sensitive yeast hosts was indeed feasible and additionally found that said introduction turned out to increase the yeast`s resistance to statins present in the relevant growth media.

Hence, to summarize, there is a need for improvement in the art of the productivity of fungal fermentations due the anti-fungal properties of statins. Drawbacks of the state of the art processes of producing statins – which are overcome by the present invention - involve *inter alia*:

(i) the fact that filamentous fungi, traditionally used for statin-production, are difficult to culture efficiently in fermenters, *inter alia* due to their unique physiology and morphology.
(ii) the potential negative effects of the traditionally used method of overexpression of HMGR (in order to reduce statin self-intoxication) might have on the central metabolism of the host microorganism
(iii) the potentially deleterious effects of the statin self-intoxication of the statin-producing host microorganisms
(iv) the contamination problems and the risk of the formation of undesirable side products associated with the traditionally used “solid state fermentation” methods of producing statins. Moreover, it is well known in the art that collecting and/or purifying the produced statin in the traditionally used “solid state fermentation” is both laborious and cost-ineffective.

Summary of the invention

The object of the present invention is *inter alia* to provide a method to solve some of the problems encountered in prior art processes of producing statins. Preferably, a process is provided which makes use of easily fermentable microorganisms, such as *Saccharomyces cerevisiae*, in which genes encoding statin efflux pumps are overexpressed.

Based on the hypothesis that the proteins MlcE, LovI/H and MokI - from the compactin, lovastatin and monacolin K gene clusters respectively - are in fact transmembrane statin specific efflux pumps the inventors of the present application have successfully expressed and overexpressed e.g. the *mlcE* gene in different *Saccharomyces cerevisiae* strains and tested the responses of said strains to increasing statin levels.

Thus, the present invention relates to the transferring of the compactin, lovastatin or monacolin K gene cluster into easily fermentable microorganisms, such as *Saccharomyces cerevisiae*, followed by overexpression of the efflux pump encoding *mlcE, mokI* and/or *lovI/H* genes in said microorganisms.

This expression or overexpression turned out to increase resistance to statins in easily fermentable microorganisms such as*, Saccharomyces cerevisiae, Pichia pastoris and Schizosaccharomyces pomp.*

Moreover, the present invention relates to the use of the transmembrane statin efflux pumps, such as MlcE, for increasing the resistance in transgenic microorganisms to the potentially deleterious effects of exogenous added statins on said microorganisms in connection with production of said statins in the organism.

This reduction of statin self-intoxication in the producing host microorganism allow for the production of elevated concentrations of natural statins compared to statin-producing methods known in the art. Moreover, overexpression in said hosts of the genes encoding the transmembrane statin efflux pumps, i.e. the *mlcE*, *lovI/H* and *mokI genes,* eliminates the potentially adverse effects of

overexpression the genes encoding the HMGR enzyme, i.e. one of the methods traditionally used in the art to produce statins in microorganisms. Moreover, expression of the mlcE, lovI/H and mokI genes increased export of statins from the cytoplasma to the growth medium, easing purification of said statins.

Definitions

Prior to discussing the present invention in further details, the following terms will first be defined. In the context of the present application, the following terms have the following meanings. The below-outlined terms are listed in alphabetical order:

Activated lovastatin: Lovastatin, as well as other statins comprise of a lactone ring unit, which can be present in an open or closed form, depending on the pH. Statins are biologically active (i.e. are able to inhibit HMGR) only when their lactone ring is in an open confirmation (dihydroxy open-acid form). Activated lovastatin is lovastatin with an open lactone ring.

ARX3 strain: *Saccharomyces cerevisiae* strain originating from CEN.PK 113-11C strain with the efflux pump encoding gene *mlcE* from the compactin biosynthetic gene cluster integrated into the genome using method described by Mikkelsen et al., 2012.

Codon optimization: A codon is a DNA entity composed of three nucleotides that is being translated into a specific amino acid residue in a polypeptide chain. The Genetic code is degenerated, meaning that many amino acids can be encoded by more than one codon. Different organisms show preferences for particular codons that encode specific amino acids. Codon optimization is a method for optimizing gene sequences in a way that the amino acid residues of the polypeptide chain are encoded by the codons preferred by the organism, in which we would like to express the gene.

Constitutive promoter (e.g. TEF1): Promoter that is active under all conditions in the cell. Gene expressed under a constitutive promoter is being continuously transcribed in the cell.

Crystal violet efflux pump (Sge1): Sge1 protein from *Saccharomyces cerevisiae* is a member of the drug-resistance protein family, and is capable of conferring resistance in yeast to crystal violet and other toxic substances.

C-terminal mRFP fusion: In order to determine subcellular localization of proteins, the proteins can be tagged with a reporter protein, e.g. red fluorescent protein (RFP) at their C terminus or N terminus. Because of the ability of RFP to emit light when illuminated with light of a specific wavelength, the fused proteins can be tracked in cells using fluorescence microscopy.

”Drug:H+ antiporter 2 family”: Drug:H+ antiporter 2 family is a family of multidrug resistance transport proteins from the major facilitator superfamily (MFS). Proteins in this family are membrane-bound enzymes containing 14 transmembrane spanning domains. They catalyze a reaction in which hydrogen protons and drugs are pumped in opposite direction across a membrane.

HC-toxin efflux pump (ToxA): ToxA protein form *Cochliobolus carbonum* is an HC-toxin efflux pump which contributes to self-protection against HC-toxin and/or secretion of HC-toxin into the extracellular environment.

HMGR: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the rate-limiting enzyme in the mevalonate pathway, which leads to the production of sterols, such as cholesterol in human, and ergosterol in fungi. The enzyme is inhibited by products from the mevalonate pathways via a negative feedback loop.

Major facilitator superfamily (MFS): MFS is a family of membrane transport proteins that facilitate movement of small molecules across cell membranes in response to chemiosmotic ion gradients.

Mevastatin: also referred to in the art as compactin (and *ML-236B*) is a hypolipidemic agent that belongs to the statins class.

MlcE topology: Topology describes the orientation of regular secondary structures, such as alpha-helices and beta strands in a protein structure and in relation to cell membranes. MlcE topology refers to the topology of the efflux pump MlcE.

mRFP: monomeric red fluorescent protein is a reporter protein used in fluorescence microscopy for subcellular localization of proteins to which mRFP is fused.

OD_{600}: Optical density (also called absorbance) is a measure of concentration of cells in a suspension. It is determined in a spectrophotometer at a wavelength of 600 nm.

Overexpression: describes the various methods by which a gene or a protein can be modified in order to increase the concentration of active enzyme, including *inter alia* (i) introduction of additional gene copies encoding host or heterologous proteins; (ii) overexpression of host proteins from a strong promoter; (iii) modifying the transcriptional regulation of the genes encoding enzymes mediating statin resistance; (iv) modifying the mRNA to increase the rate of translation initiation; (v) mutation of critical amino acids leading to proteins with improved kinetic properties; (vi) mutations causing an increased half-life of the enzyme; (vii) modifying the mRNA molecule in such a way that the mRNA half-life is increased; Other methods which are well-known in the art may be envisaged.

pdr5 deletion strain (Pleotropic Drug Resistance gene): the *pdr5* gene encodes a pump that has shown to confer a basic level of statin-resistance in *Saccharomyces cerevisiae. The pdr5* deletion strain (herein denoted as: *pdr5*Δ) does not contain said pump.

Plate dilution assay (spot assay): This assay allows testing of the toxic effects of the compounds added to a solid growth medium. It is based on culturing a dilution series of a microorganism on said plates, following the growth of a microorganism and observing at which dilution the microorganism is unable to grow. The growth of individual microorganisms as a function of time is recorded by photography of the plates.

Penicillium citrinum: the compactin-producing filamentous fungi (also referred to in the art as *Penicillium solitum*)

Recombinant host strains: refers to host strains in which genetic material from one or multiple sources have been brought together, creating sequences that would not otherwise be found in biological organisms.

RFP-tagged MlcE: To investigate subcellular localization of efflux pump MlcE, the relevant protein has been fused with RFP at its C terminus.

Standard Protein BLAST: Basic Local Alignment Search Tool is an algorithm for comparing biological sequence information. Standard Protein BLAST, available at e.g. http://www.ncbi.nlm.nih.gov is commonly used for identifying a query amino acid sequences in protein databases. The search tool is designed to identify local regions of similarity.

Substantially homologous polynucleotide: A polynucleotide with nucleotide sequences that are substantially homologous to a reference sequence is defined as a polynucleotide with a nucleotide sequence with a degree of identity to the specified nucleotide sequence of at least 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, still more preferably at least 97%, still more preferably at least 98%, most preferably at least 99%

Substantially homologous polypeptide: A polypeptide with amino acid sequences that are substantially homologous to a reference sequence is defined as a polypeptide with an amino acid sequence with a degree of identity to the specified amino acid sequence of at least 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, still more preferably at least 97%, still more preferably at least 98%, most preferably at least 99%. Substantially homologous polypeptides may for example contain only conservative substitutions of one or more amino acids of the specified amino acid sequences or substitutions, insertions or deletions of non-essential amino acids.

TEF promoter: constitutive promoter that regulates transcription of the 'Transcription Elongation Factor b' encoding gene

TOPCONS server: TOPCONS server is a tool for consensus prediction of membrane protein topology. It is available online at http://topcons.net/

Wild type strain: Reference *Saccharomyces cerevisiae* strain, in this study CEN.PK 113-11C (MATa MAL2-8C SUC2 his3Δ ura3-52).

YPD agar plates: YPD is a complete medium for yeast growth composed of yeast extract, peptone and glucose. If YPD is used as a solid medium, agar is added to YPD, and the medium is solidified in plates for cultivation of microorganisms.

Advantages of the present invention

The inventors of the present application surprisingly showed that it is possible to use the transmembrane statin efflux pumps MlcE, LovI/H and MokI as a resistance mechanism, i.e. for reducing the potentially deleterious effects of self-intoxication caused by the produced statins in a statin-producing microorganism, e.g. in yeasts such as *Saccharomyces cerevisiae*. This is a surprising finding in light of the prior art which e.g. suggested that this could not be the case as expression of the *mlcE* gene in e.g. the filamentous fungus *Aspergillus nidulans*, which is normally sensitive to statins, did not increase its resistance against the tested compounds (see e.g. the article by Hutchinson et al., 2000).

An additional advantage of the present invention, in addition to providing resistance against both natural and semi-statins, is that the statin efflux pumps also provides an elegant solution for exporting the produced statins into the extracellular medium in statin-producing hosts other than filamentous fungi, e.g. in yeasts such as *Saccharomyces cerevisiae.*

Furthermore, by expressing e.g. the *mlcE* gene in easily fermenting hosts such as *Saccharomyces cerevisiae* there is no longer a need for the traditionally used overexpression of HMGR which in turn eliminates the potential negative effects that HMGR might have on the central metabolism of the host microorganism.

The above-mentioned statin pumps, e.g. MlcE, with their ability of exporting natural and semi-natural statins across the plasma membrane has a great

potential for improving a statin-producing yeast cell factory. Not only do said pumps, e.g. MlcE, provide the resistance to a range of statins in yeast; it also ensures the export of the produced statins into the extracellular environment, which can significantly ease the subsequent purification of the produced compounds compared to the traditionally used "solid state fermentation" methods based on naturally producing species of *Penicillium*, *Aspergillus* and *Monascus.*

Hence, the inventors of the present application provided evidence indicating that the polypeptides encoded by the *mlcE, LovI/H* and *MokI* genes are transmembrane efflux pumps capable of transporting both natural and semi-natural statins out of the statin-producing host cell.

Moreover, the inventors of the present application showed that MlcE, LovI/H and MokI are statin-specific transporters, with the ability to transport compactin as well as the compactin-related compounds lovastatin, simvastatin and pravastatin, across the plasma membrane. Therefore, in light of the above, overexpression of e.g. *mlcE* in statin-producing microorganisms, such as *Saccharomyces cerevisiae* could greatly improve the commercial production of natural and semi-natural statins compared to well-known statin-producing methods.

In general this means that the statin efflux pumps MlcE, LovI/H and MokI provide resistance to both, natural and semi-natural statins, making them great tools for optimizing e.g. yeast cell factories for statin production.

Detailed description of the invention

Thus, it is an object of the present invention to provide a statin producing method and statin producing transgenic, non-filamentous microorganisms that solves the above mentioned problems of the prior art.

Thus, one aspect of the invention relates to a method for the production of statin in a transgenic microorganism, wherein the method comprises expression in said microorganism of one or more polynucleotide(s) encoding one or more transmembrane statin efflux pump(s).

In a preferred embodiment, the polynucleotide(s) of said method are chosen from the group consisting of SEQ ID NOs: 1, 2, 3, 4 and/or 5.

In a further preferred embodiment, the polynucleotide(s) of said method is/are chosen from the group consisting nucleotide variants comprising sequences with a degree of identity to any of SEQ ID NOs: 1, 2, 3, 4 and/or 5 of at least: 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, still more preferably at least 97%, still more preferably at least 98%, most preferably at least 99%.

In an even further preferred embodiment, the polynucleotide(s) SEQ ID NOs: 1, 2, 3, 4 and/or 5 of said method is/are overexpressed.

In an even further preferred embodiment, the above-mentioned polynucleotide variant(s) of SEQ ID NOs: 1, 2, 3, 4 and/or 5 is/are overexpressed.

Another aspect of the invention relates to a method for the production of statin in a transgenic microorganism, wherein the method comprises expression in said microorganism of polypeptide(s) chosen from the group consisting of SEQ ID NOs: 17, 18, 19 and/or 20.

In a further preferred embodiment, the polypeptide(s) of said method is/are chosen from the group consisting of variants comprising sequences with a degree of identity to any of SEQ ID NOs: 17, 18, 19 and/or 20 of at least: 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, still more preferably at least 97%, still more preferably at least 98%, most preferably at least 99%.

In an even further preferred embodiment, the polypeptide(s) of SEQ ID NOs: 17, 18, 19 and/or 20 of said method is/are overexpressed.

In an even further preferred embodiment, the above-mentioned polypeptide variants of SEQ ID NOs: 17, 18, 19 and/or 20 is/are overexpressed.

In an even further preferred embodiment, the transgenic microorganism for use in the production of statins is a non-filamentous fungus selected from the group consisting of *Saccharomyces cerevisiae, Pichia pastoris* and *Schizosaccharomyces pomp.*

In an even further preferred embodiment, the present invention relates to statins produced by any of the above methods.

Another aspect of the present invention relates to transgenic microorganism for use in the production of statin, wherein the microorganism comprises one or more polynucleotide(s) encoding one or more transmembrane statin efflux pump(s).

In a preferred embodiment, said transgenic microorganism comprises one or more polynucleotide(s) chosen from the group consisting of SEQ ID NOs: 1, 2, 3, 4 and/or 5.

In a further preferred embodiment, the transgenic microorganism comprises one or more nucleotide variant(s) comprising sequences with a degree of identity to any of SEQ ID NOs: 1, 2, 3, 4 and/or 5 of at least: 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, still more preferably at least 97%, still more preferably at least 98%, most preferably at least 99%.

In an even further preferred embodiment, the transgenic microorganism comprises one or more polynucleotide(s) according to SEQ ID NOs: 1, 2, 3, 4 and/or 5 which is/are overexpressed.

In an even further preferred embodiment, the transgenic microorganism comprises one or more of the above polynucleotide variants of SEQ ID NOs: 1, 2, 3, 4 and/or 5 which are overexpressed.

In a preferred embodiment, said transgenic microorganism comprises one or more polypeptides chosen from the group consisting of SEQ ID NOs: 17, 18, 19 and/or 20.

In a further preferred embodiment, the transgenic microorganism comprises one or more polypeptide variant(s) comprising sequences with a degree of identity to any of SEQ ID NOs: 17, 18, 19 and/or 20 of at least: 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, still more preferably at least 97%, still more preferably at least 98%, most preferably at least 99%.

In an even further preferred embodiment, the transgenic microorganism comprises one or more polypeptide(s) according to SEQ ID NOs: 17, 18, 19 and/or 20, which is/are overexpressed.

In an even further preferred embodiment, the transgenic microorganism comprises one or more of the above polypeptide variants of SEQ ID NOs: 17, 18, 19 and/or 20, which is/are overexpressed.

In an even further preferred embodiment, the above transgenic microorganism is a non-filamentous fungus selected from the group consisting of *Saccharomyces cerevisiae, Pichia pastoris* and *Schizosaccharomyces pomp.*

Yet another aspect of the present invention relates to use of a transmembrane statin efflux pump in a transgenic non-filamentous statin-producing microorganism, for the production of statins in the microorganism and/or increasing the statin resistance in the microorganism and/or decreasing the statin self-intoxication in the microorganism and/or increasing the export of the produced statins into the extracellular medium.

Also envisaged is the use of the statins obtained in any of the above-outlined methods in the production of a medicament.

A further aspect of the invention concerns a use of a transmembrane statin efflux pump in a microorganism, for the:
(i) bioconversion of statins in the microorganism and/or
(ii) increasing the statin resistance of the microorganism and/or
(iii) increasing the export of statins into the extracellular medium

An even further aspect of the invention concerns a polypeptide selected from the group consisting of SEQ ID NOs: 17, 18, 19 and/or 20 which, when incorporated into a transgenic non-filamentous microorganism, is capable of
(ii) providing statin resistance in the microorganism and/or
(ii) exporting of the produced statins out of the microorganism

Also envisaged is one or more polypeptide variant(s) comprising a sequence with a degree of identity to any of SEQ ID NOs: 17, 18, 19 and/or 20 of at least: 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, still more preferably at least 97%, still more preferably at least 98%, most preferably at least 99% which, when incorporated into a transgenic non-filamentous microorganism, is capable of
(ii) providing statin resistance in the microorganism and/or
(ii) exporting of the produced statins out of the microorganism

A further aspect of the invention concerns a nucleic acid construct comprising any of the polynucleotide sequence according to SEQ ID NOs: 1, 2, 3, 4 and/or 5 operably linked to one or more control sequences that facilitate production of the polypeptide in an expression host.

An even further aspect of the invention concerns a recombinant expression cassette comprising said construct either maintained in the expression host as a self-replicating plasmid or integrated into the genome.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

Brief description of the figures and the SEQ ID NOs

Figure 1:
Phylogenetic tree of representative members of the two families of MFS drug resistance proteins; family DHA1 with Drug:H+ antiporters consisting of 12 TMS and family DHA2 with Drug:H+ antiporters consisting of 14 TMS are shown.The three putative statin efflux pumps (MlcE, LovI and MkI from compactin, lovastatin

and monacolin biosynthetic gene clusters, respectively) are predicted to belong the DHA2 family. Protein sequences were obtained from UniProt Knowledgebase (UniProtKB, http://www.uniprot.org/help/uniprotkb), aligned with multiple sequence alignment tool MAFFT version 7 (Multiple sequence Alignment using Fast Fourier Transform) available at the European Bioinformatics Institute (http://mafft.cbrc.jp/alignment/server/). The tree was generated with ClustalW2 alignment program at EMBL-EBI using Neighbor-Joining clustering method (Setting: distance correction on, exclude gaps on), and viewed with FigTree software, version 1.4 (http://tree.bio.ed.ac.uk/software/figtree).

Figure 2:

Strain construction and subcellular localization of MlcE-RFP. A) Schematic representation of strain construction. Expression cassettes containing mlcE and its RFP-tagged version were integrated into the genome, under the control of the constitutive promoter TEF1 (strains ARX3 and ARX1, respectively). RFP alone has been expressed from the same locus and promoter, and the resulting strain (strain ARX2) was used as a control for fluorescence microscopy. B) Fluorescence microscopy results. For subcellular localization of the putative efflux pump MlcE strain ARX1, and a control strain ARX2 were incubated overnight in 10 mL of SC medium, shaking (150 rpm) at 30°C. Images obtained by differential interference contrast microscopy (DIC) (left panel) and corresponding fluorescence images (right panel) are shown.

Figure 3:

Investigation of the potential of MlcE to confer the resistance to statins in yeast. Tenfold dilution series of *Saccharomyces cerevisiae* WT and ARX3 strain harbouring the putative efflux pump MlcE, starting with an OD_{600} of 0.02 were prepared from overnight cultures. 4.5 microliters of each dilution were plated on a set of YPD agar plates containing different cytotoxic compounds. The plates were incubated at 30°C for 2 days, after which the growth of the strains was recorded by photography.

Figure 4:
Investigation of the potential of MlcE to complement the PDR5 efflux pump in yeast. Fivefold dilution series of *Saccharomyces cerevisiae* WT, ARX3, AR29 pdr5Δ and pdr5Δ strains, starting with an OD_{600} of 0.2 were prepared from overnight cultures. Four microliters of each dilution were plated on a set of YPD agar plates with increasing concentration (0.74 mM and 1.98 mM) of lovastatin. The plates were incubated at 30°C for 3 days, after which the growth of the strains was recorded by photography.

SEQ ID NO: 1 represents the nucleotides of *mlcE* (coding sequence, from the compactin biosynthetic gene cluster (GenBank accession number: AB072893.1))

SEQ IN NO: 2 represents the nucleotides of *mlcE* (coding sequence, synthetic codon optimized version)

SEQ ID NO: 3 represents the nucleotides of *mlcE-mRFP* (coding sequence, synthetic codon optimized version of *mlcE* with mRFP fusion)

SEQ ID NO: 4 represents the nucleotides of *lovI/H* (coding sequence (GenBank accession number: AF141925.1))

SEQ ID NO: 5 represents the nucleotides of *mokI* (coding sequence (GenBank accession number: DQ176595.1))

SEQ ID NO: 6 represents the nucleotides of the primer mlcE-F

SEQ ID NO: 7 represents the nucleotides of the primer mlcE-R

SEQ ID NO: 8 represents the nucleotides of the primer TEF1-d

SEQ ID NO: 9 represents the nucleotides of the primer PGK1-s

SEQ ID NO: 10 represents the nucleotides of the primer RFP_F+

SEQ ID NO: 11 represents the nucleotides of the primer RFP_R+

SEQ ID NO: 12 represents the nucleotides of the primer mlcE-RFP-R

SEQ ID NO: 13 represents the nucleotides of the primer RFP-F

SEQ ID NO: 14 represents the nucleotides of the primer C1_TADH1_F

SEQ ID NO: 15 represents the nucleotides of the primer PDR5-DEL-F

SEQ ID NO: 16 represents the nucleotides of the primer PDR5-DEL-R

SEQ ID NO: 17 represents the amino acids of MlcE (GenBank accession number: BAC20568.1)

SEQ ID NO: 18 represents the amino acids of LovI/H (GenBank accession number: AAD34558.1)

SEQ ID NO: 19 represents the amino acids of MokI (GenBank accession number: ABA02247.1)

SEQ ID NO: 20 represents the amino acids of MlcE-mRFP

The invention will now be described in further details in the following non-limiting examples.

Example 1 (integration of the *mlcE* gene into *Saccharomyces cerevisiae)*

General setup

The *mlcE* gene was codon optimized and expressed from a genomic locus in *Saccharomyces cerevisiae* as a single copy gene under the control of a strong constitutive promoter (TEF1). The gene was introduced into a 'wild type' strain and a *pdr5* deletion strain (*pdr* = Pleotropic Drug Resistance gene). The *pdr5* gene encodes a pump that has shown to confer a basic level of statin-resistance in *Saccharomyces cerevisiae* (Hirata & Yano, 1994; Riccardo & Kielland-Brandt,

2011). Furthermore, it has previously been shown that elimination of the *pdr5* gene sensitize the strain in question which, in turn, allows for a larger dynamic test range with respect to statin effects.

The efflux pump encoding gene *mlcE* was integrated into a defined locus of *Saccharomyces cerevisiae*, CEN.PK 113-11C (MATa MAL2-8C SUC2 his3Δ ura3-52), genome using a yeast expression platform established by Mikkelsen et al. 2012

The yeast strain CEN.PK 113-11C (*MATa MAL2-8C SUC2 his3Δ ura3-52*) was donated by Dr. Petter Kötter, Institut für Mikrobiologie, der Johan Wolfgang Goethe-Universität, Frankfurt am Main, Germany*. Escherichia coli*, DH5□, was used to propagate the plasmids.

The media

Yeast strains were cultivated in standard liquid yeast peptone dextrose (YPD) or synthetic complete (SC) medium. Yeast transformants were selected on SC medium lacking uracil. Removal of the URA3 marker, via direct repeat recombination, was achieved by growing the strain on SC medium containing 740 mg/L 5-fluororotic acid (5-FOA) and 30 mg/L uracil. The *E. coli* transformants were selected on LB medium containing 100 µg/mL ampicillin.

The inventors of the present application then tested yeast susceptibility to statins and statin-unrelated compounds by growing yeast strains on solid YPD medium supplemented with compactin, lovastatin, simvastatin, pravastatin sodium, atorvastatin, mycophenolic acid (MPA) or vanillin respectively. Vanillin, MPA and atorvastatin stock solutions were prepared by dissolving the compounds in 99.9% ethanol followed by filter-sterilization. Compactin, lovastatin, and simvastatin were converted to their active forms.

More specifically, the solid compounds were dissolved in 1 mL 99% ethanol, preheated to 50°C, alkalinized with 0.5 mL of 0.6 M NaOH and incubated at 50°C for 2 hours. The pH of the solutions was then adjusted to 7.2 by adding 0.4 M HCl. Final volume of all the solutions was adjusted to 2 mL with water, resulting in stock solutions of 50 mM. The statin stock solutions were filter-sterilized and

stored at -20°C. Compactin and atorvastatin were purchased from Toronto Research Chemicals, lovastatin from Tokyo Chemical Industry, MPA and vanillin from Sigma-Aldrich, and simvastatin was purchased from Ark Pharm.

Compound	Concentration of the stock solution [mM]	Source
Compactin	50	Toronto Research Chemicals (Canada, Ontario, Toronto)
Lovastatin	50	Tokyo Chemical Industry (Japan, Tokyo)
Simvastatin	50	Ark Pharm (USA, Illinois, Libertyville)
Atorvastatin	10	Toronto Research Chemicals (Canada, Ontario, Toronto)
Vanillin	30	Sigma-Aldrich (USA, Missouri, St. Louis)
MPA	320	Sigma-Aldrich (USA, Missouri, St. Louis)

Plasmid construction

The *mlcE* gene was codon-optimized for expression in *Saccharomyces cerevisiae* (by the company Evolva). The codon-optimized version of the *mlcE* gene was amplified from plasmid pEN669 (source: Evolva) with primers mlcE-F and mlcE-R. Together with the TEF1 promoter, the amplified gene was cloned into the X-3 vector via USER cloning technique, resulting in plasmid pX3-TEF1-mlcE. To determine the intracellular localization of MlcE, a red fluorescent protein (RFP) was fused to its C-terminus.

For that plasmid pX3-TEF1-mlcE-RFP and a control plasmid pX3-TEF1-RFP were constructed: *mlcE* without the stop codon was amplified from plasmid pEN669 using primers mlcE-F and mlcE-RFP-R, and RFP was amplified from plasmid pWJ1350 using either RFP-F (for tagging mlcE) or RFP_F+ (for the control plasmid) and RFP_R+ primers. All fragments were amplified by PCR using a USER cloning compatible PfuX7 polymerase.

Table: List of plasmids used

Name	Description	Reference
pEN669	mlcE template	Purchased from Evolva
pWJ1350	RFP template	Lisby et al. 2003
pSP-G2	PGK1,TEF1 template	Partow et al. 2010
pX3 TEF1 mlcE	Plasmid carrying a gene-targeting cassette for expressing mlcE in yeast.	This study
pX3 TEF1- RFP	Plasmid carrying a gene-targeting cassette for expressing RFP-tagged mlcE in yeast.	This study
pX3 TEF1 mlcE RFP	Plasmid carrying a gene-targeting cassette for expressing RFP in yeast.	This study

Strain construction

The constructed plasmids were digested with the NotI restriction enzyme (purchased form New England Biolabs), and the linear fragments were used for yeast transformation using the lithium acetate/polyethylene glycol/single carrier DNA transformation method. The URA3 marker in all the constructed strains was excised by direct repeat recombination, and the correct integrations of the gene were verified by colony PCR with one primer annealing in the yeast genome next to the integration site, and one primer annealing inside the introduced DNA.

Targeted deletion of the pleiotropic drug resistance pump (*pdr5*) encoding gene in the reference and X3::*TEF1-mlcE* expressing strains was performed, as described by Güldener et al 1996, using the primers PDR5-DEL-F and PDR5-DEL-R.

Table: list of strains used

Name	Genotype	Reference
CEN.PK113-11C	MATα MAL2-8C SUC2 his3Δ ura3-52	Kindly donated by Dr. Petter Kötter, Institut für Mikrobiologie, der Johan Wolfgang Goethe-Universität, Frankfurt am Main, Germany
ARX3	MATα MAL2-8C SUC2 his3Δ ura3-52 X3(pTEF1-mlcE)	This study
pdr5Δ	MATα MAL2-8C SUC2 his3Δ ura3--52 pdr5Δ	This study
AR29pdr5Δ	MATα MAL2-8C SUC2 his3Δ ura3-52 X3(pTEF1-mlcE)	This study
ARX1	MATα MAL2-8C SUC2 his3Δ ura3-52 (pTEF1-mlcE-RFP)	This study
ARX2	MATα MAL2-8C SUC2 his3Δ ura3-52 (pTEF1-RFP)	This study

Example 2 (phylogenetic tree)

An initial sequence comparison investigation of the putative efflux pump MlcE from the compactin biosynthetic gene cluster using Standard Protein BLAST showed that this protein strongly resembles some of the known export proteins from the major facilitator superfamily (MFS), such as crystal violet efflux pump Sge1 from *Saccharomyces cerevisiae* and HC-toxin efflux pump ToxA from *Cochliobolus carbonum.* Moreover, prediction of MlcE topology using the TOPCONS server suggested that MlcE comprises 14 transmembrane-spanning regions (TMS), possibly classifying MlcE to the Drug:H+ antiporter 2 family (DHA2; 14 TMS) of the MFS drug transporters, a family which ToxA and Sge1 belong to as well. The inventors of the present application constructed a phylogenetic tree, which suggests that MlcE, together with its orthologs from the lovastatin and monacolin biosynthetic gene clusters, LovI and MkI, respectively, does indeed belong to the DHA2 family of drug resistance proteins with 14 TMS (Figure 1).

Table: Proteins used for the phylogenetic tree construction

Protein	**Microorganism**	**Representative Substrate**	**Accession Number (UniProtKB)**
MFS-DHA2 (14 TMS)			
ToxA	*Cochliobolus carbonum*	HC-toxin	Q00357
Tri12	*Fusarium sporotrichioides*	Trichotechene	O93842
CFP	*Cercospora kikuchii*	Cercosporin	O93886
Atr1	*Saccharomyces cerevisiae*	Aminotriazole	P13090
Sge1	*Saccharomyces cerevisiae*	Crystal violet	P33335
EmrB	*Escherichia coli*	CCCP [b]	P0AEJ0
LfrA	*Mycobacterium smegmatis*	Acriflavin	Q50392
QacA	*Staphylococcus aureus*	Benzalkonium chloride	Q1XG09
SmvA	*Salmonella typhimurium*	Ethidium bromide	P37594
ActVa 1	*Streptomyces coelicolor*	Actinochodrin	Q53903
CmcT	*Nocardia lactamdurans*	Cephamycin	Q04733
Mmr	*Streptomyces coelicolor*	Methylenomycin A	P11545
Pur8	*Streptomyces lipmanii*	Puromycin	P42670
MFS - DHA 1 (12 TMS)			
Ctb4	*Cercospora nicotinae*	Cercosporin	A0ST42
CefT	*Acremonium chrysogenum*	Cephalosporin	Q8NKG7
Mdr1	*Canidida albicans*	Fluconazole	P28873

Flu1	*Candida albicans*	Fluconazole	G1UB37
Bcr	*Escherichia coli*	Bicyclomycin	C6EA15
Blt	*Bacillus subtilis*	Acriflavin	M1U4Q0
EmrD	*Escherichia coli*	CCCP [b]	P31442
CaMDR1	*Candida albicans*	Benomyl	Q9URI2
NorA	*Staphylococcus aureus*	Acriflavin	P0A0J7
CyhR	*Candida maltosa*	Cycloheximine	P32071
CmlA	*Pseudomonas aeruginosa*	Chloramphenicol	Q83V15
Flr1	*Saccharomyces cerevisiae*	Fluconazole	P38124
Tpo1	*Saccharomyces cerevisiae*	Spermine	Q07824
Dtr1	*Saccharomyces cerevisiae*	Dityrosine	P38125
Aqr1	*Saccharomyces cerevisiae*	Quinidine	P53943
Statin Efflux pumps - unknown family			
MlcE	*Penicillium citrinum*	Compactin	Q8J0F3
LovI	*Aspergillus terreus*	Lovastatin	Q9Y7D4
MkI	*Monascus pilosus*	Monacolin	Q3S2U5

Example 3 (toxicity analysis on dilution tests)

The constructed strains response to different lovastatin levels present in the growth medium were tested using a agar-plate dilution assay (also known as a spot assay). Overnight cultures of the four *Saccharomyces cerevisiae* strains (wt, ARX3, AR29 *pdr5*Δ, *pdr5*Δ,) were diluted to OD_{600} of 0.2 and a fivefold dilution series for each strain was made. Four microliters of each dilution were deposited on a series of agar plates with different concentrations of activated lovastatin (0 mM, 0.74 mM, 1.98 mM). The idea behind this assay is that it allows for

reproducible testing of toxic effects by observing at which dilution steps the different strains are able to form visible colonies, under a given concentration of the toxic compound. The growth of the individual strain as a function of time was recorded by photography.

The plate assay (figure 4) confirmed that the *pdr5*Δ strain is more sensitive to lovastatin than the wild type (wt), as evidenced by the lack of growth even at the lowest tested concentration (0.74 mM). Expression of the *mlcE* gene allows both the wild type and *pdr5*Δ strain to grow at elevated statin concentrations and at the higher dilutions evidencing that the MlcE efflux pump indeed can provide statin resistance in yeast cells, such as *Saccharomyces cerevisiae*.

Example 4 (subcellular localization of the MlcE efflux pump)

To determine the subcellular localization of the MlcE efflux pump *in Saccharomyces cerevisiae* and in order to test the hypothesis that the MlcE protein is in fact a transmembrane efflux pump, the inventors of the present application constructed a C-terminal mRFP fusion and expressed it from the same locus in *Saccharomyces cerevisiae*. The resulting strain was analyzed by fluorescent microscopy and compared to a *Saccharomyces cerevisiae* strain that expressed mRFP (cytoplasmic localization) from the same promoter and the same locus as the strain with the RFP-tagged MlcE.

More specifically, the MlcE was tagged with RFP at the C- terminus, and integrated into the previously described site in the yeast genome under the control of TEF1 promoter, resulting in the yeast strain ARX1 (Figure 2A). Fluorescent microscopy of ARX1 revealed a ring-like distribution of fluorescence around the cell (Figure 2B top panel), indicating that the tagged putative efflux pump was localized in the plasma membrane. In contrast, the mRFP alone was found to have a uniform cytoplasmic distribution in the control cells ARX2 (Figure 2B bottom panel), expressing RFP alone from the same locus and controlled by the promoter as in strain ARX1. These results support the prediction that MlcE is a trans-membrane protein and shows that the protein is targeted to the plasma membrane in *S. cerevisiae*.

Moreover, to determine if the putative efflux pump MlcE has the ability to export statins across the plasma membrane the inventors of the present application also tested whether MlcE confers resistance to statins in yeast. To achieve that, *mlcE* was expressed from a defined genomic locus in *Saccharomyces cerevisiae* as a single copy gene under the control of a strong constitutive promoter pTEF1 (Figure 2A). The resulting yeast strain ARX3 was tested for susceptibility to compactin by serial dilution plating of both wild type (WT) and ARX3 strains on YPD agar plates supplemented with the active form of compactin (Figure 3). The efflux pump harbouring strain ARX3 showed an increased resistance to compactin present in the medium compared to the wild type strain, suggesting that MlcE is indeed a compactin efflux pump capable of exporting this natural statin out of the cells and not into storage compartments such as the vacuole.

Example 5 (the MlcE pump in *Saccharomyces cerevisiae* confers resistance against other types of statins)

The inventors of the present application additionally showed that *Saccharomyces cerevisiae* strains with the inserted transmembrane efflux pump MlcE had an increased resistance not only to compactin but also to the other natural statin, lovastatin, when compared to the wild type yeast strain.

In addition to this, the inventors of the present application surprisingly found that introduction of the transmembrane efflux pump MlcE into *Saccharomyces cerevisiae* strains also resulted in increased resistance to the semi-natural statin simvastatin, when compared to the wild type yeast strain.

In contrast, MlcE does not seem to have the ability to export compounds, which are structurally unrelated to its natural substrate compactin, namely atorvastatin, vanillin and mycophenolic acid (MPA) because ARX3 strain does not show an increased resistance to these compounds (Figure 3).

On this basis the inventors of the present application concluded that MlcE is not a multi-drug resistance efflux pump such as for example Pdr5 and Sge1 from *Saccharomyces cerevisiae* but rather a transmembrane statin specific efflux pump.

In general this means that the efflux pump MlcE provides resistance to both, natural and semi-natural statins, making it a great tool for optimizing yeast cell factories for statin production.

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Claims

1. Method for the production of statin in a transgenic microorganism, wherein the method comprises expression in said microorganism of one or more polynucleotide(s) encoding one or more transmembrane statin efflux pump(s).

2. Method according to claim 1, wherein the polynucleotide(s) are chosen from the group consisting of SEQ ID NOs: 1, 2, 3, 4 and/or 5.

3. Method according to claim 2, wherein the polynucleotide(s) comprise(s) one or more nucleotide variant(s) comprising sequences with a degree of identity to any of SEQ ID NOs: 1, 2, 3, 4 and/or 5 of at least: 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, still more preferably at least 97%, still more preferably at least 98%, most preferably at least 99%.

4. Method according to any of claims 2 or 3, wherein one or more of the polynucleotide(s) according to SEQ ID NOs: 1, 2, 3, 4 and/or 5 is/are overexpressed and/or wherein one or more of the polynucleotide variant(s) according to claim 3 are overexpressed.

5. Method according to any of claims 1-4, wherein the transgenic microorganism is a non-filamentous fungus.

6. Method according to claim 5, wherein the non-filamentous fungus is selected from the group consisting of *Saccharomyces cerevisiae, Pichia pastoris* and *Schizosaccharomyces pomp.*

7. Statins produced by a method according to any of claims 1-6.

8. Transgenic microorganism for use in the production of statin, wherein the microorganism comprises one or more polynucleotide(s) encoding one or more transmembrane statin efflux pump(s).

9. Transgenic microorganism according to claim 8, wherein the polynucleotide(s) is/are chosen from the group consisting of SEQ ID NOs: 1, 2, 3, 4 and/or 5.

10. Transgenic microorganism according to claim 9, wherein the polynucleotide(s) comprises one or more nucleotide variant(s) comprising sequences with a degree of identity to any of SEQ ID NOs: 1, 2, 3, 4 and/or 5 of at least: 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, still more preferably at least 97%, still more preferably at least 98%, most preferably at least 99%.

11. Transgenic microorganism according to any of claims 9 or 10, wherein one or more of the polynucleotide(s) according to SEQ ID NOs: 1, 2, 3, 4 and/or 5 is/are overexpressed and/or wherein one or more of the polynucleotide variants according to claim 10 is/are overexpressed.

12. Transgenic microorganism according to any of claims 8 or 11, wherein the transgenic microorganism is a non-filamentous fungus.

13. Transgenic microorganism according to claim 12, wherein the non-filamentous fungus is selected from the group consisting of *Saccharomyces cerevisiae, Pichia pastoris* and *Schizosaccharomyces pomp.*

14. Use of a transmembrane statin efflux pump in a transgenic non-filamentous statin-producing microorganism, for the:
(i) production of statins in the microorganism and/or
(ii) increasing the statin resistance in the microorganism and/or
(iii) decreasing the statin self-intoxication in the microorganism and/or
(iii) increasing the export of the produced statins into the extracellular medium

15. Use of the statins obtained in any one of claims 1 to 7 in the production of a medicament.

Abstract

The present invention relates e.g. to methods of producing statins in transgenic, non-filamentous microorganisms such as *Saccharomyces cerevisiae*. In addition, the present invention relates to the transgenic, non-filamentous microorganisms *as such* as well as various uses of transmembrane statin efflux pump(s) originating from various filamentous fungi. Moreover, the present invention relates to the transferring the compactin, lovastatin or monacolin K gene cluster originating from non-filamentous fungi into easily fermentable microorganisms, followed by expression or overexpression of the efflux pump encoding genes in said microorganisms in order to increase the microorganisms resistance to statins which in turn allows for production of elevated concentrations of natural statins compared to statin-producing methods known in the art.

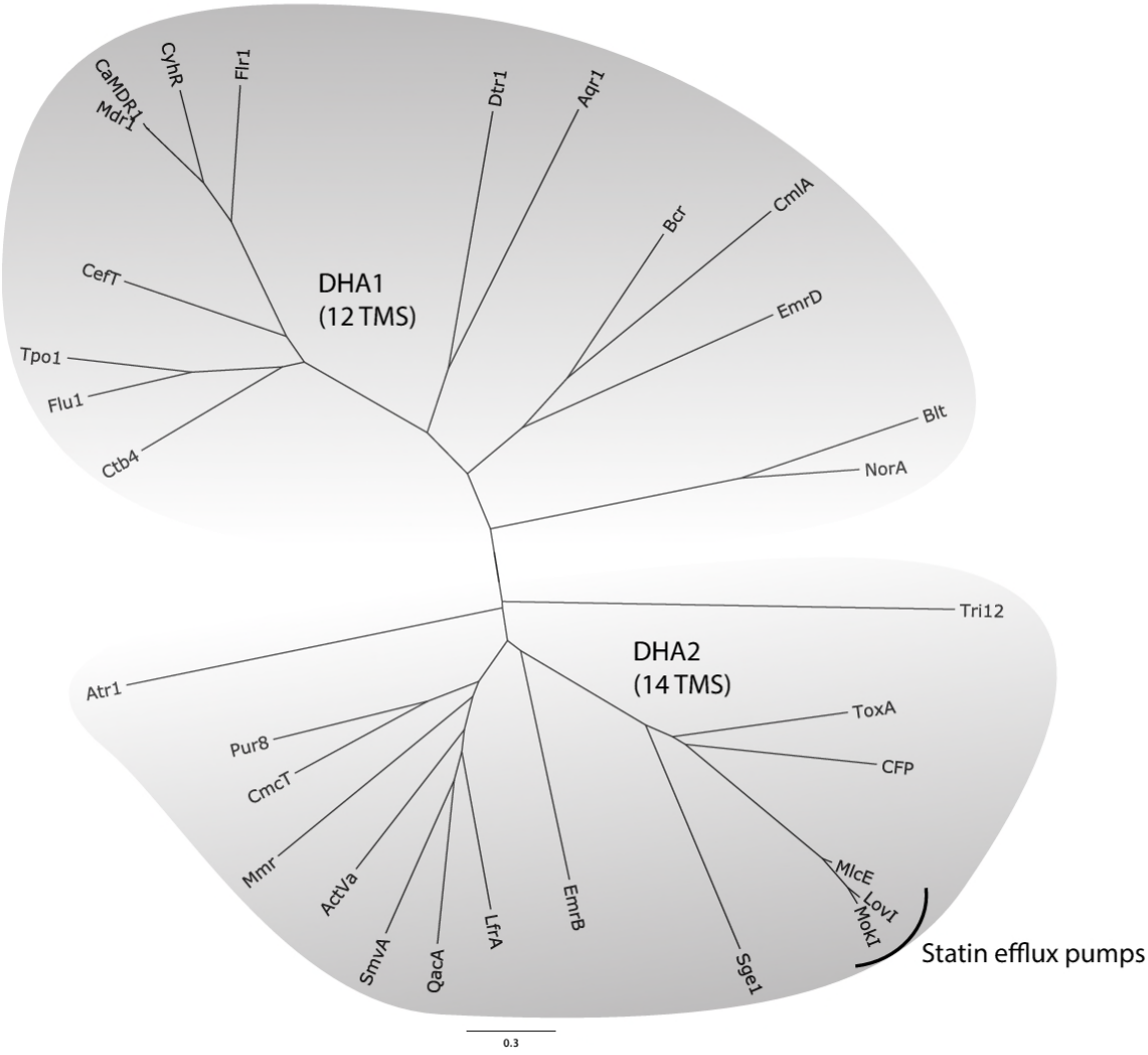

Figure 1:
Phylogenetic tree

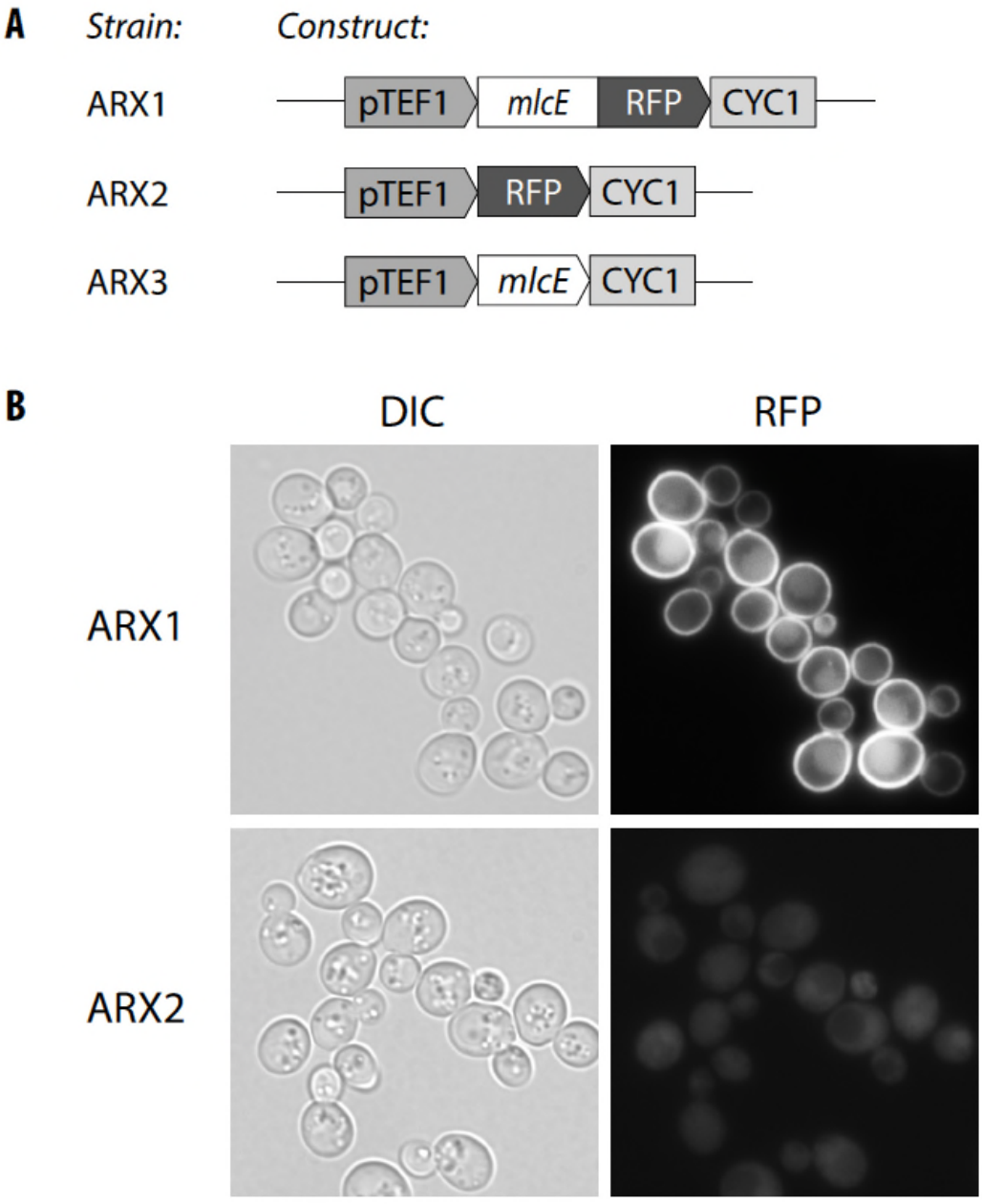

Figure 2:
Strain construction and subcellular localization

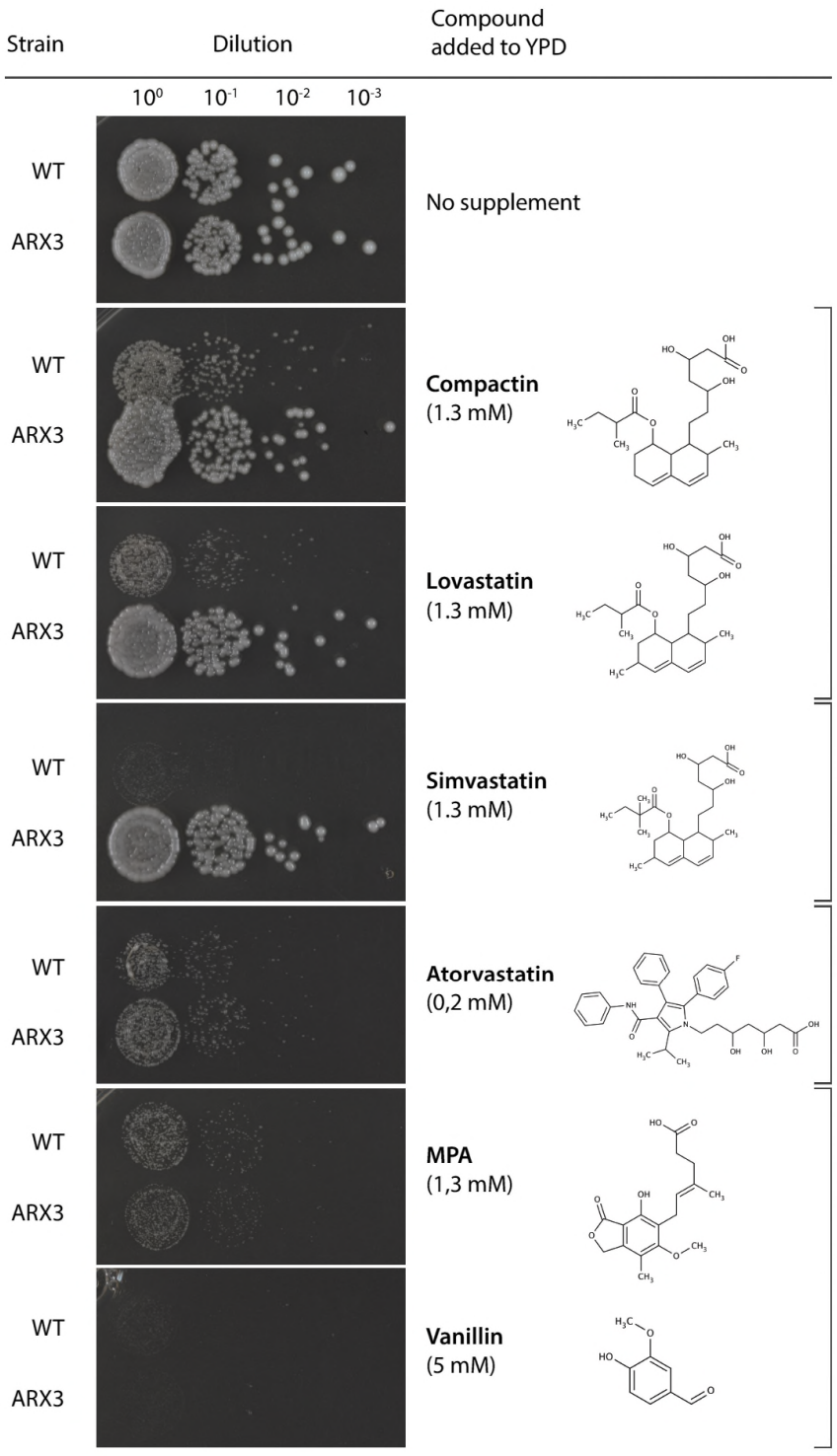

Figure 3:
Investigation of the potential of MlcE to confer the resistance to statins in yeast

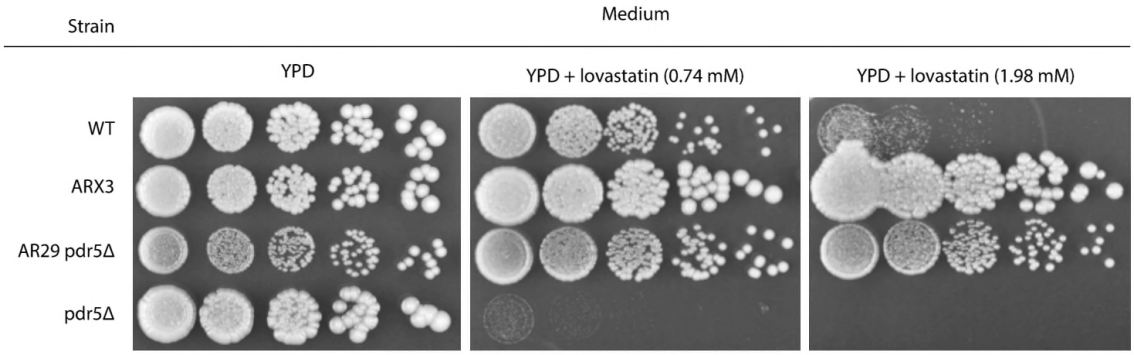

Figure 4:
Investigation of the potential of MlcE to complement the PDR5 efflux pump in yeast